

Neutralizing antibodies against Chikungunya virus and structural elucidation of their mechanism of action

Corresponding Author: Professor Jinghua Yan

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Dear Authors,

This is an important and newsworthy report that will further antibody based therapies for an important viral disease. Strengths of this article is a direct utility for translation to a potential monoclonal antibody therapy and may inform vaccine design. The article is well written and the methods are generally logical and well-presented. The methods are rigorous including analysis of treatment and prophylactic dosing.

Given there is currently a licensed live attenuated chikv vaccine and a VLP vaccine almost approved that will provide protection at a fraction of the cost of monoclonal antibody therapies, it should be discussed the significance of the discovery of these monoclonal antibody properties in the context of these vaccines.

I suggest a description of why an immunodeficient mouse model was selected when immunocompetent mouse models are available that perhaps better recapitulate human disease that is generally not lethal.

The issue of viral evolution and the likelihood of conservation of effectiveness of these mAbs should be discussed.

Reviewer #2

(Remarks to the Author)

// SUMMARY //

This study by Han et al. seeks to identify and characterise (incl. structurally) neutralising-protective antibodies against CHIKV. They do this using a combination of in vitro biochemistry, virology, and structural biology. Their principal finding is that specific monoclonal antibodies have a 'triple' mode of action targeting the interaction of CHIKV with its putative receptor MXRA8.

This is a large paper, and there is a lot of data contained within. I do not doubt the effort on the part of the authors. However, I do think some conclusions are questionable and substantial improvements really do need making.

One major criticism is that the paper is very difficult to follow in several areas. In particular, I think the structural biology elements of the paper could benefit from being significantly re-written as it is sometimes impossible to work out which structure is being referred to at any one time. There is also a lot of information missing from the methods and materials part of the manuscript.

I think the authors could do more to highlight what exactly is novel in this work when compared to other recent papers, like Zhou et al. 2020 (PNAS), who reported similar data looking at two other CHIKV monoclonal antibodies. Separately, I remain unconvinced in relation to the data looking C34/C37 and MXRA8 blocking.

I make further comments in relation to the science in the paper in the following sections. I hope that these comments are helpful.

// RESULTS //

For the in vivo protection studies on mice, the authors plot survivability. Did they evaluate virus presence/load in different biological niches/organs? Can they speculate on C34/C37's ability to protect against virus dissemination, for example?

The authors used exclusively female IFNAR1-KO mice (according to the methods). It is well-established that female mice mount greater humoral and cell-mediated immune responses to several infections and other immunologic challenges. How therefore do they account for sex differences?

For this reviewer, the text relating to the VLP/mAbs structures is incredibly difficult to wade through and needs significant re-writing. By my count, the authors refer to up to 11 reconstructions for 3 conditions: VLP (iso, 2f, and 5f), VLP+C34 (iso, 2f, 3f, and 5f) and VLP+C37 (iso, 2f, 3f, and 5f). However, for this reviewer, it is seldom clear which reconstruction the authors are referring to. Further, the figures that support this section, while visually are generally very nice, do not actually help unpick the results as they are presented. The text itself only refers to figures typically once (in the opening statements) and then never again, which is not helpful.

Line 188-189, the authors state that they used locally filtered maps to account for resolution decay in the Fab region. Yet, this does not appear in the methods. This is important detail to include as local filtering, in particular for EM maps.

Line 189-190, the authors state that "fully occupied Fab densities were observed at the 2f, 3f, and 5f vertices..." What do the authors mean by this? That the Fabs are fully there (i.e. fit'able) in all reconstructions? Because this certainly not the case.

A non-exhaustive list of things for the authors to check in relation to their structural data: -

In the 2f_block_C37VLP structure, long stretches of Fab are missing/not supported by the provided maps. In Chain O, this is from residue 120 onwards. In chain P, this is from residue 110 onwards.

In the C34_E1E2 structure, volume for residue ~290 onwards in chain B is missing and yet something has been fit.

In the X-ray crystal structure, denoted C37_refine_5, there is no believable 2Fo for residue 290 onwards in chain F, and yet something is fit here. Could the authors provide difference maps for this area?

The authors state that they used "localized 3D classification" to explore whether broken density was due to partial occupancy or high flexibility. Separately, they indicate processing was conducted in cryoSPARC. However, the reference to 'T' values, which are used by Relion leads me to question this. What did they actually do here?

I think also that the question the authors posed is never actually answered. They found a single class incl. 95% of particles, and this showed 5 Fabs bound around the 5f vertex. The authors were also able to fit 5 Fabs bound in this area without direct clashes. So why would 5 Fabs binding at this site in this way cause instability? Maybe it does but it's not clear that there's a structural basis for that conclusion.

The comparison between chains is highly problematic here. The text reads: "The S26 residue of the C34 light chain was in close proximity to the CH domain of a neighbouring Fab." If you look at the structure, not only is there no convincing volume for the S26 chain at a reasonable rmsd itself but the closest the sidechain is to neighbouring chains is either 12 Å (chain G) or 16 Å (chain Q).

This is important as it is a key argument made for "a weak but widespread crosslinking effect among neighbouring Fabs, potentially enhancing multivalent effects and neutralization potency." In my view this, conclusion is not backed fully by the data contained within the manuscript.

The authors explore the potential for interviriion crosslinking by dynamic light scattering (DLS) finding that the IgG form of C34/C37 induced dramatic increases while the Fab forms did not. In Figure 3m, the authors plot the single condition tested for this, however, for this reviewer, this is insufficient. To really explore the impact of the C34/C37 Fab/IgG on VLP crosslinking, testing a series of molar ratios would be helpful. This would allow the authors to show convincingly that with an increase in Fab/IgG, you see fold-changes in VLP crosslinking.

For this reviewer, the section on C34/C37 blocking MXRA8 attachment very confusing. The authors state that "docking MXRA8 and the corresponding Fabs onto ... these three interfaces revealed direct steric hindrance at interfaces..." Except, the figure supporting this assertion shows some protein domains overlapping with a squiggly line.

The authors state that all Fab fragments overlap with the MXRA8 stalk region, but again, I cannot really see this from the figures provided.

I really do not understand 5f and 5g. What are they telling us? The text in the manuscript is just not helpful in this section.

The hypothesis, as far as I understand it, is that the antibodies bind to the spike, and that this then precludes binding of the

MXRA8 stalk region to the spike. I am not convinced, however, that this is proven by the data here. To me, it would seem fairly straight forward to test, however, by designing a MXRA8 construct missing the stalk region? Even if this is just in vitro, you could then design an assay to explore the interaction of the spike, MXRA8 (or not, as the case should be), and antibody?

// METHODS //

There is a general lack of information relation to the E1-E2 expression and purification.

Into which plasmid was the expression construct cloned, and how? How did the authors harvest the Hi5 cells and into which buffer? How were the cells lysed and supernatant clarified?

What buffers were used for the His-tag purification? What buffers were used for the gel filtration step?

The authors tell us that the E1-E2 fusion protein was further 'purified' using an ion-exchange column without providing any information on this. It should be provided.

How was the fusion protein concentrated and stored?

In relation to the crystallisation, it is not clear to me how the authors prepared the C37 Fab-CHIKV E protein complex. They reference procuring the complex from gel filtration but the only reference to gel filtration in the methods is in relation to the E1-E2 fusion construct and not a C37 Fab?

Where did the protein come from for the complex, and what was the stoichiometry of the complex? The authors also indicate that the complex was concentrated. Is that correct, the i.e. the reconstituted complex was concentrated or was each component concentrated separately and then mixed together?

The authors tell us that initial crystals were obtained using "several similar conditions." They should just tell us what those are, providing ranges, if necessary.

Can the authors reflect on what "initial crystals" to "improved crystals" actually means? Were they (more convincingly) single? Well-defined? Large? What was it that the authors were seeking to improve?

How did the authors determine space group? Which programme? This should be clarified and referenced.

The paper reference for the molecular replacement search model should be included at line 602.

I am not sure what the authors mean by showing "good stereochemical quality" in relation to the Ramachandran plot. There is no agreed target for favoured/allowed/outliers.

I cannot find a Table S1 including data on the crystal structure. This is a major omission. For reference, as far as I can tell, there is only a Table S1, which references exclusively the cryo-EM structures included in the paper.

I have several questions relating to the cryo-EM sample preparation. What were the Vitrobot parameters used during grid preparation? In particular, temperature, humidity, blotting time, and blotting force.

It is considered good practice, where you have multiple different data collections to split these and report the magnification, defocus range, and total dose for each. This information is currently mixed in the methods.

The authors state that particles were picked from micrographs using Topaz. How and on what data did they train Topaz?

References are missing for programmes used in lines 676-685, in particular, MolProbity and Rosetta.

// FIGURES //

Fig 1b – this reviewer finds it incredibly hard to work out which curve on the greyscale is what – not accessible at all.

Figure 2 – for the survival data, it is very difficult to see what data points are statistically significant by comparison to each other. The authors may wish to consider picking 1 or 2 key timepoints, let's say dpi 3 and dpi 15, and then plotting the data only for those timepoints.

Figure 4 – it would be useful for the authors to indicate a database ID for the provided virus sequences.

Figure 5 – I have made several comments in relation to this figure set above, I will not repeat them here.

We are missing any text for the Figure S7 and S8 figure legends.

(Remarks to the Author)

Brief summary:

Han et al. describe the isolation and characterization of two neutralizing and protective anti-chikungunya virus (CHIKV) human monoclonal antibodies, C34 and C37, from a convalescent patient of CHIKV infection. Through in vitro studies, the authors determine that C34 and C37 inhibit viral entry through aggregation of virus particles and blockade of Mxra8 binding. Structural analyses elucidated the mechanism of C34 and C37 Fab binding to an epitope within β -ribbon cleft on the E2 glycoprotein, a region normally occupied by the E3 protein. However, there are major points that are missing to provide a greater impact to the field. Furthermore, the novelty of the manuscript is moderate as previous studies have shown similar epitopes and neutralization mechanism(s) of action. To improve the manuscript, the authors should address the corresponding comments.

General concept comments:

The authors define the E2 β -ribbon cleft as a rarely studied epitope. However, several reports describe this epitope and the neutralization mechanism(s) of action similar to those described here (Quiroz et al., 2019 [PMID: 31697791], Zhou et al., 2020 [PMID: 33087569], Raju et al., 2023 [PMID: 37196061]). The authors state that this is an “unique” (line 91) or “novel” (line 339) epitope, however given these previous studies, this epitope does not appear “unique”. Furthermore, several examples of mAbs and their corresponding epitopes are shown in Figure 4F and stated that CHK-263 “binds to an identical epitope” (line 322). This epitope is also not “unique” to CHIKV but to other alphaviruses, such as WEEV (SW24; Sutton et al., 2023 [PMID: 37295404]). Can the authors remove the “unique” aspect of C34 and C37 and provide a description of these previous studies in the introduction? It would also be helpful to include a more detailed discussion comparing C34 and C37 to these previously described mAbs (CHK-263, CHK-152, 516.C10, 516.H09, 506.C07, etc.).

The authors describe in the introduction that “how these antibodies affect CHIKV’s interaction with its receptor remain poorly understood.” However, multiple antibodies have been described that block Mxra8 binding and are not referenced in the proper context of the corresponding manuscript: “Zhang et al., 2018 [PMID: 29769725], Zhou et al., 2020 [PMID: 33087569], Powell et al., 2020 [PMID: 32783883], Raju et al., 2023 [PMID: 37196061].” Can the authors please describe how their studies differ from these previous studies and expand on the “novelty” of the data generated in this manuscript? It would be helpful to compare the Mxra8 blocking activity of C34 and C37 to these previously described mAbs.

Can the authors provide a better description of the E2-E1 fusion protein (EFL) and the E protein? The methods are not very detailed. Does this refer to p62E1, E2-E1 heterodimer, or full-length envelope protein? Usually, the proteins are referred to as the corresponding E2 or E1 proteins, p62E1, E2-E1 heterodimer, or virus-like particle (VLP).

The authors observe binding of C34 or C37 to the E2-E1 heterodimer but weak binding to monomeric E2 or E1 protein. However, the authors showed data that supports C37 binding to the CHIKV E2 protein. Please explain this discrepancy. Furthermore, the weaker binding could be explained by a conformational difference in display of the epitope between the heterodimer and monomeric proteins. Please include a discussion about this.

The flow cytometric cell sorting data of CHIKV-reactive IgG⁺ B cells are missing. Please include the data in the manuscript.

The authors state that “blocking cell attachment is a key mechanism of neutralization for C34 and C37” (lines 138-139). However, the relatively strong neutralization activity of C34 and C37 in the post-attachment assay indicates that C34 and C37 do not prevent virus attachment as a main neutralization mechanism of action. This may in part contribute as a mechanism of action. However, given the modest neutralization activity of C34 and C37 this indicates otherwise. Please modify the conclusion appropriately.

The described “triple-action” mode for viral entry inhibition has been described before. To further define the neutralization mechanism(s) of C34 and C37, additional studies are needed. For example, do C34 and C37 inhibit viral fusion, egress, or cell-to-cell spread? Please perform these experiments as they are readily established in the field.

The in vivo studies are not described very well. Please expand on these studies. Why were mice aged 8-12 weeks old chosen (usually 4–5-week-old mice)? Footpad injections in immunocompromised models are generally lower viral titers (101 to 103 FFU). What strain of CHIKV was used? Were the mice challenged by footpad or subcutaneous injection? These are used interchangeably. Please keep consistent as “subcutaneous inoculation of the footpad”. Was a negative control mAb (instead of PBS) included for comparison? Did the authors correct for multiple comparisons in statistical analyses?

The authors suggest that full occupancy is achieved in the observed reconstruction. However, this is an average of particles. Can the authors please expand on why full occupancy would be observed? Furthermore, as there are residues within proximity of each other (lines 211-213) and the authors describe a potential “cross-linking effect among neighboring Fabs” (line 215), would an IgG be able to bind due to the presence of the Fc region? Please include a section about this in the results and discussion.

The authors observed distances <50 Å between neighboring Fab molecules of C34 and C37, which enables intra-virion cross-linking as previously described for EEEV Fab molecules. However, when assessing for aggregation of VLPs by dynamic light scattering, the authors observed significant aggregation, which suggests inter-virion cross-linking. However, these observations counteract each other. At what VLP:IgG molar ratio was the DLS experiment performed? If the authors

titrate the molar ratio, are there differences in the aggregation potential of C34 and C37?

The authors describe residues N245 and D250 (line 269) as pivotal for C34 and C37 Fab molecule binding. How did the authors come to this conclusion? Mutagenesis or viral escape studies are warranted to understand the contribution of these residues for Fab/IgG binding and neutralization.

In this manuscript, the authors state that a reconstruction of infectious CHIKV particles was generated. However, there is no reference to these data in the manuscript. Can the authors please include these data? Furthermore, PDB ID: 6NK7 was used to build an atomic model (line 289). However, this also contains the mouse Mxra8 receptor, which may yield an inappropriate initial model to build upon.

The authors describe that C34 and C37 “inhibit the exposure of the fusion loop” (line 344). Can the authors please expand on how they came to this conclusion? It would be helpful to include studies assessing fusion inhibition.

In the discussion, the authors state that the mAbs isolated from the CHIKV patient have different mechanism(s) of action compared to those elicited by CHIKV VLP vaccination. However, Raju et al., 2023 [PMID: 37196061] isolated and characterized mAbs that recognize similar epitopes, potentially neutralize, and protect against CHIKV infection. Can the authors please provide a more direct comparison to this publication? Furthermore, this is a strong statement with little evidence to support this and requires additional studies.

The methods could provide more detail. Please include additional information, including strains used, assay details, etc.

Specific comments:

In the results, the first referenced figure legend corresponds to a supplemental figure (Figure S1). The figures should be referenced in order, starting with Figure 1. Please change accordingly.

The authors refer to “ONNV E protein as having the highest sequence similarity with CHIKV-E” (lines 121-122). However, as referenced in Figure 1a or S2 or anywhere else within the manuscript, the analysis is not provided. Figure 4F provides an alignment of the E2 epitope. However, the level of conservation is unclear. Can the authors please provide a sequence similarity analysis of the corresponding viruses and proteins?

Figure 1: Were positive and negative control mAbs included? Please show if so.

Figure 1B: Please show the error bars.

Figure 1D-1E: Please keep the axes consistent for better comparison of mAb activity.

Figure 2 Legend Title: Remove “Pre- and post-attachment assays and”.

Figure 2C and 2G: Please keep the axes consistent.

Figure 2D: Please correct y-axis label.

Figure S6: Please include the FSC for VLP alone reconstructions.

Figure 3: It would be useful to include a RIVEM image of the relative binding epitope for C34 and C37.

Figure 4A-4B: Please change the color of E2 β -ribbon region. It is hard to distinguish between the C34 Fab molecule and this domain.

Title: Chikungunya virus should be all lowercase.

Line 24: Please change to “illness” instead of “infections”.

Line 36: Can the authors explain what they mean by the clash between the antibodies and the stalk region of Mxra8? What is the “stalk region” referring to?

Lines 38-39: Please remove “as a common and pivotal target for antibodies in the same class as C34 and C37” and add “... cryo-EM reconstructions with C34 and C37 identify ...”

Line 41: Please remove “functional and structural” to improve conciseness

Line 55: Please change “E3-E2” to “p62” for consistency.

Line 56: Please keep the proteins in order of the polyprotein reading frame. Change “C, E1, p62, and 6K” to “C, p62, 6K, E1” and “E2 and E3” to “E3 and E2”.

Lines 62-66: Discuss the role the E2 protein plays in preventing premature exposure of the fusion loop.

Line 63: Please remove “E2-“ for conciseness. Define DA, DB, DC.

Line 65: Please remove “domains” if the authors want to use DA and DB for conciseness.

Line 68: Please change “fusion peptide” to “fusion loop”.

Lines 124-125: Please include the data not shown for Figure S2.

Line 127: “In vitro” should all be lowercase.

Line 146: “In vivo” should all be lowercase.

Lines 143-144: Please expand on the following statement, “this phenotype indicates that the attachment-inhibiting activities are strongly associated with the Fab regions of the two mAbs”.

Line 181: Add “... , respectively, ...” before “... for C34 Fab bound ...”.

Lines 182-183: Were reconstructions of the 3f blocks for VLP alone determined?

Lines 184-185: What is the RMSD value upon C34 and C37 binding?

Line 221: Please describe which cysteine residue was used to measure distances between Ca atoms. Cys216?

Lines 250-259: It would be helpful to include a table listing the corresponding residues instead for conciseness.

Lines 370-371: Please avoid use of the word “synergy” but instead rely on “cooperative”, since synergy requires more precise definition.

Line 391: Please change “pr” to “pM”.

Please change “MXRA8” to “Mxra8”, unless the authors are discussing the gene. There are many instances of this, but as examples, lines 35, 36, 71, 92, etc.

Please change “Fabs” to “Fab molecules”. There are many instances of this, but as examples, lines 171, 175, 184, etc.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The responses to my review were adequate thank you.

Reviewer #2

(Remarks to the Author)

// Summary //

One of my major criticisms was that the paper was very difficult to follow in several areas. In particular, the structural biology elements of the paper. I think this has improved but there are still areas where I get quite lost.

The findings remain the same in so far that specific monoclonal antibodies apparently have a ‘triple’ mode of action targeting the interaction of CHIKV with its putative receptor Mxra8. I remain skeptical however, that the blocking/specific interaction with Mxra8 is demonstrated by the data presented in the manuscript, and I explore this below too.

In my initial review, I said that I thought the authors could do more to highlight what exactly is novel in this work when compared to other recent papers, like Zhou et al. 2020 (PNAS). Other reviewers also highlighted this as a concern.

While I acknowledge and appreciate that the results of the paper has seen significant re-writing, the discussion of the manuscript remains untouched and so the novelty question remains. There is a lot of data in this manuscript. The authors really must reflect on how their studies different from previous studies and expand on the novelty of the data presented here and, importantly, include those reflections in the manuscript itself.

As before, I make further comments in relation to the science in the paper in the following sections. I hope that these comments are helpful.

// Structural Biology //

Line 200. Can I suggest to the authors that they include somewhere a simplified diagrammatic representation of the virus capsid proteins? If you are unfamiliar with the various components, it is very easy to get lost in Figure 3. What is E1 vs. E2, for example? I realise this is explained in the introduction but seeing this makeup would make things easier to understand.

Line 215-219. I still find all the different resolutions confusing. Can you put these into a small table? This would also simplify the text greatly... 'An overview of reconstructions and their calculated overall resolutions is provided in Table X.'

Line 228-232. The authors tell us that the binding of the C34 and C37 fabs do not alter overall virus assembly. This would be a fairly straightforward finding to show in a supplementary figure showing the overall diameters unchanged between the VLP alone and VLP/Fab reconstructions.

Line 233. I think the authors mean "asymmetric unit" rather than each "asymmetry unit."

Line 260-262. 'The S26 residue of the C34 light chain was in close proximity to the CH domain of a neighboring Fab, while the R218 residue of the C37 heavy chain was near the Q156 residue of a neighboring Fab's light chain (Fig. 3m).' You cannot see this in the figure as it is currently drawn.

Line 274-275. 'For C37 Fabs Fab molecules around the 2f vertex, all measured distances were within the 50 Å theoretical radius for intravirion crosslinking (Fig. 3g-h, k-l).' I still find this very confusing. Which figure should I be looking at? The figure legend doesn't help me decode what is going on in Figure 3. If I look at Figure 3g, then the distances are all greater than this theoretical limit?

Line 266-294. Can I suggest to the authors that they sequester this part of the text in its own little sub-section? It reads well and is reasonably self-contained.

Line 200-265. I still find this overview complicated and confusing. I think the authors have made improvements, which I greatly appreciate. But I still tend to think that it's perhaps overly verbose and makes limited use of figures. As far as I can tell there is a single reference to a supplementary figure?

In my view, the manuscript could use reading by perhaps a collaborator unfamiliar with the experiments to really address some of the difficulties with language.

Line 348. 'After block-based reconstruction,' is an incomplete sentence.

Line 346-369. This part is quite nice to read through but again, there is a lot happening and the authors describe fairly dynamic processes. The only figure referenced is Figure 4e and I am not really sure what that tells me. Can the authors think about ways in which they can better show the dynamism, for example, of the D250 sidechain and its impact on neighbouring residues?

Line 398. What do the authors mean by 'global domains'?

In response to my comments indicating that various bits of protein are missing from the provided maps, the authors indicate that they used locally-filtered maps to fit these parts of the protein. The authors should make this absolutely clear in the manuscript, specifically what experimental maps have been used to produce the deposited 3D structure.

The authors offer to upload a 2Fo map for the X-ray crystal structure, this would be helpful to see.

Line 371-425. I remain unconvinced at this stage that the title of this section that 'C34 and C37 block the Mxra8 attachment by interfering with the stalk region' is clearly demonstrated by the data shown. See specific comments below.

Figure 5b-d remains confusing to me and, I suspect, others. I understand what it is meant to show, the outcome of protein-protein docking experiments. However, I still do not understand why the authors cannot simply show these experiments? If the authors wish to rely on in silico docking/prediction then they surely have to show this data. For readers, it will be important to see where and how the Mxra8 stalk region sits within the spike. Currently, this is represented by an orange squiggly line, which is not the same thing. Please show the outputs from your docking experiments. Further, as far as I can tell, there is little reference to docking experiments in the methods?

Line 378-381. The authors tell us that docking experiments showed no direct steric hindrance between C34 or C37 and the Mxra8 within a given stalk. But we cannot see this in the figure.

Line 381-383. The authors tell us that there may be "potential clashes" between the Fabs and Mxra8 bound to adjacent spikes. Again, we cannot see this in the figure. The authors should also clarify what they mean by "potential clashes". What does this mean in practice (i.e. lengths, distances, etc.)? This detail is important particularly if this is the only mode of inhibition (remembering that we have just been told that within a spike there is no steric hindrance).

Line 383-385. The authors tell us that they analysed "three distinct interfaces" when in Figure 5b-d there are 4 interfaces reported? Can the authors show, specifically and unambiguously what interfaces 1, 2, 3, and 4 refer to somewhere? So that this is absolutely clear to readers.

Line 387-390. The authors tell us 'Docking Mxra8 and the corresponding Fab molecules onto these three interfaces revealed direct steric hindrance at interfaces 2, 3, and 4 for the C34 Fab, while no apparent clash was observed for other docked interfaces (Fig. 5b-d).' However, we cannot actually see this in practice in the figure. See comments above.

Line 389-390. I take specific interest in this comment '... no apparent clash was observed for other docked interfaces.' I am not sure that is true based on the figure legend for Figure 5. The figure legend tells us that a red asterisk indicates an "apparent clash", a red triangle indicates a "clash" and red circle indicates a "potential clash". So based on this categorisation, actually there are clashes reported for interface 1 (C34), and interfaces 2, 3, and 4 (C37). Potential clashes exist in other cases.

// Methods //

I thank the authors for now including the classic "Table 1" for their X-ray crystal structure. However, I note several discrepancies between the table and the manuscript text.

For example, the data collection wavelength is different (0.97915 in the paper vs. 0.97853 in the table). Similarly, the final Rwork and Rfree values are different (for Rwork, the paper has 0.239 whereas the table has 0.2216). The Ramachandran values are also different. For example, 0.43% of residues are outliers in the paper, 0% are outliers in the table.

I suggest the authors double-check the figures in the paper and table in relation to their X-ray crystallography experiments.

In my initial review, I left this comment: "It is considered good practice, where you have multiple different data collections to split these and report the magnification, defocus range, and total dose for each. This information is currently mixed in the methods." To be clear, by this I meant... where you have multiple data collections (with different parameters, though, not always) pooled into a single dataset. Does this apply here?

// Figures and Tables //

I think the two supplementary tables are missing legends. These should be included allowing the authors to clarify how key metrics were calculated, for example, Rwork in the case of their X-ray crystallography experiments.

Supplementary Table 1. The map resolution range for all reconstructions is missing. Ditto for model resolution range, although, I am not sure how these would really be different here?

Reviewer #4

(Remarks to the Author)

I've been asked to comment on the revisions performed to Reviewer #3's concerns.

The authors have adequately addressed many of the points raised by Reviewer #3, however, some issues require clarification.

Key Remaining Issues:

1. The main point of novelty in this manuscript is the claim that C34 and C37 block MXRA8 engagement by blocking the stalk. However, the data supporting this claim remain problematic. It is clear neutralizing mAbs do not share overlapping epitopes with MXRA8, and blocking effects seen in other studies may simply be due to non-specific steric blockade.

- A negative control mAb which does not "block the stalk" would help validate the specificity of the assay.
- It is clear between Stalk (N-tag) and no stalk (N-tag) that the MFI of no stalk is higher possibly explaining diminished "blocking" activity observed with the mAbs. The same is true between Stalk C-tag and GS C-tag (1 log higher MFI). These should be titrated so that there is similar staining in the control so blocking activity can be more directly compared.
- The data may be more clear by titration of the blocking mAb as well.
- Quantification of the assay by MFI is necessary along with appropriate statistics.
- The section in the results describing the experiments performed are quite difficult to parse through.

2. The title of Fig.4 claims D250 is a key binding site, however the authors own mutagenesis shows mutation of D250 has minimal to no effect on binding. The reviewer recognizes this is a site of overlap between both mAbs, but the authors have not demonstrated this is a critical residue that mediates binding. The claim should be tempered or re-written.

3. The "inhibition of the fusion loop" is a strong claim. The FFWO assay simply shows the antibodies block fusion. I don't think the authors can really make the claim that these antibodies block exposure of the fusion loop. The claims should be tempered.

Minor:

- Does the CHK-263 epitope exactly overlap with C34 and C37? While C34 and C37-like antibodies have been characterized, a possible point of novelty that could be highlighted.
- The construct encoding the E1-E2 FL protein is still quite unclear, the citation in the method refers to the Song et al Cell

2019 paper which used p62-E1. Could the authors expand the methods to describe the exact residues and linkers used to construct this molecule.

3. The flow cytometry plots would benefit from labelling of what molecule is being measured in addition to fluorochrome used to help orient the reader.

Version 3:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors have addressed most of my comments. I have one small and final suggestion in relation to a previous comment about the theoretical limits for intravirion crosslinking.

Line 287-289. 'For C37 Fab molecules around the 2f vertex, all measured distances were within the 50 Å theoretical radius for intravirion crosslinking while slightly above 50 Å for C34 (Fig. 3g-h, k-l,k).'

Can I suggest that the authors include a reference here introducing this theoretical radius for intravirion crosslinking either in the case of CHIKV or some other related virus? Or describe how they calculate the number themselves.

Reviewer #4

(Remarks to the Author)

The manuscript is improved.

I appreciate the dose-titration of mAb blockade and transparency of data display—however again in Fig 5E (left two panels) the MFI between no stalk and stalk are markedly different. The right two panels showing stalk and GS-linker show more similar MFI of binding, and here the difference in blockade is reduced. Statistics are still missing.

The text is still in need of major improvement.

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Thank you again for submitting your manuscript "Identification of Neutralizing Antibodies Against Chikungunya Virus and Structural Elucidation of Their Triple-action Mode for Viral Entry Inhibition" to Nature Communications. We have now received reports from 3 reviewers and, after careful consideration, we have decided to invite a major revision of the manuscript.

As you will see from the reports copied below, the reviewers raise important concerns. We find that these concerns limit the strength of the study, and therefore we ask you to address them with additional work. Without substantial revisions that address the reviewers concerns regarding the structural conclusions, binding mode and technical aspects, we will be unlikely to send the paper back to review.

If you feel that you are able to comprehensively address the reviewers' concerns, please provide a point-by-point response to these comments along with your revision. Please show all changes in the manuscript text file with track changes or colour highlighting. If you are unable to address specific reviewer requests or find any points invalid, please explain why in the point-by-point response.

We identified additional blob densities above the E2 domain B and the β -ribbon connecting E2 domains A and B (namely the A-B strand) at both the 5-fold and 2-fold axes, revealing the definite shape of Fabs on both VLP structures complexed with C34 and C37.

Reviewer #1 (Remarks to the Author):

Dear Authors,

This is an important and newsworthy report that will further antibody based therapies for an important viral disease. Strengths of this article is a direct utility for translation to a potential monoclonal antibody therapy and may inform vaccine design. The article is well written and the methods are generally logical and well-presented. The methods are rigorous including analysis of treatment and prophylactic dosing.

Thank you for your insightful and constructive feedback. We appreciate your recognition of the significance of our work, particularly its potential impact on antibody therapies and vaccine development for chikungunya virus, as well as your positive comments on the clarity and rigor of our methods.

We thank you for raising important points regarding the therapeutic use of monoclonal antibodies and the choice of immunodeficient mouse models. We will address all points and modify the manuscript accordingly.

Given there is currently a licensed live attenuated chikv vaccine and a VLP vaccine almost approved that will provide protection at a fraction of the cost of monoclonal antibody therapies, it should be discussed the significance of the discovery of these monoclonal

antibody properties in the context of these vaccines.

We have modified the introduction accordingly, and added the contents below: "While vaccines offer population-wide protection and are essential for preventing infection, mAbs have unique advantages in specific therapeutic contexts, such as post-exposure prophylaxis, treatment for immunocompromised individuals, and the potential for personalized medicine. Additionally, monoclonal antibodies can target highly conserved epitopes, offering protection against emerging variants."

I suggest a description of why an immunodeficient mouse model was selected when immunocompetent mouse models are available that perhaps better recapitulate human disease that is generally not lethal.

Thank you for your insightful suggestion. While immunocompetent mouse models can indeed be used for CHIKV infection studies, as demonstrated in the 2015 Cell article (PMID: 26553503), immunodeficient mouse models exhibit more pronounced symptoms post-challenge, providing clearer and more objective endpoints for evaluation. In contrast, disease manifestations in immunocompetent mice tend to be more variable and subjective.

Since our primary goal was to assess the protective efficacy of antibodies, we aimed to use a model with well-defined and measurable outcomes. Given that immunodeficient mice provide a lethal challenge model, we believe this approach is more suitable for evaluating antibody-mediated protection in our study.

The issue of viral evolution and the likelihood of conservation of effectiveness of these mAbs should be discussed.

Thank you for your suggestion. The C34 and C37 antibodies are supposed to cross-react with ONNV, an alphavirus holding very high conservation with CHIKV. In response, we have included a comparative sequence alignment of different CHIKV strains as well as other representative alphaviruses to illustrate the conservation of the virus. This figure is now presented in Fig S1. We also added a short description of the sequence alignment result in the section of antibody epitope analysis.

Reviewer #2 (Remarks to the Author):

// SUMMARY //

This study by Han et al. seeks to identify and characterise (incl. structurally) neutralising-protective antibodies against CHIKV. They do this using a combination of in vitro biochemistry, virology, and structural biology. Their principal finding is that specific monoclonal antibodies have a 'triple' mode of action targeting the interaction of CHIKV with its putative receptor MXRA8.

This is a large paper, and there is a lot of data contained within. I do not doubt the effort on

the part of the authors. However, I do think some conclusions are questionable and substantial improvements really do need making.

One major criticism is that the paper is very difficult to follow in several areas. In particular, I think the structural biology elements of the paper could benefit from being significantly re-written as it is sometimes impossible to work out which structure is being referred to at any one time. There is also a lot of information missing from the methods and materials part of the manuscript.

I think the authors could do more to highlight what exactly is novel in this work when compared to other recent papers, like Zhou et al. 2020 (PNAS), who reported similar data looking at two other CHIKV monoclonal antibodies. Separately, I remain unconvinced in relation to the data looking C34/C37 and MXRA8 blocking.

I make further comments in relation to the science in the paper in the following sections. I hope that these comments are helpful.

We thank the reviewer for the constructive criticism and the points raised especially on structural analysis, neutralizing mechanism, and the concerns on MXRA8 blocking. We have addressed all the points raised by the reviewer and have modified text in the updated manuscript accordingly. Detailed responses are listed below.

// RESULTS //

For the in vivo protection studies on mice, the authors plot survivability. Did they evaluate virus presence/load in different biological niches/organs? Can they speculate on C34/C37's ability to protect against virus dissemination, for example?

Thank you for your insightful comment. In addition to the survival curves, we also measured foot swelling and body weight. These metrics are widely used and reliable indicators for evaluating the protective effect of antibodies for CHIKV infection. Given that the primary aim of our study is to assess the protective effect of the antibodies, we chose to focus on foot swelling as a clear and measurable endpoint. Moreover, due to the joint tropism of CHIKV, studying antibody protection in the footpads is a rational approach.

While viral load and tissue damage data could provide additional insights, survival curves and foot swelling measurements already provide a comprehensive evaluation of antibody protection. Additionally, conducting experiments to measure viral load in tissues would require extensive time and resources in a P3 laboratory, which are often limited and require scheduling over prolonged periods. Considering these practical constraints, we concluded that supplementing with additional animal experiments might not be necessary for the scope of this study.

For the ability of C34 and C37 to protect against virus dissemination, however, we performed in vitro FFWO experiments that illustrated their activity to block fusion. Please find results in Fig 1. of revised manuscript.

The authors used exclusively female IFNAR1-KO mice (according to the methods). It is well-established that female mice mount greater humoral and cell-mediated immune responses to several infections and other immunologic challenges. How therefore do they account for sex differences?

Thank you for raising this important point. We agree that sex differences in immune responses are well-established, and the potential impact of these differences on our study should be considered. However, since the antibodies specifically target the virus, we believe that hormonal levels difference upon virus challenge are unlikely to significantly affect their protective efficacy. In our experiments, all mice in the control group died, while all mice in the antibody protection group survived. Additionally, we included a survival curve for non-neutralizing antibody C59, which also showed mortality, confirming the reliability of the model in evaluating antibody protection. Due to potential aggression among male mice, which necessitates separate housing, and considering animal ethics restrictions. Therefore, it is unnecessary to include male mice in the experiments.

For this reviewer, the text relating to the VLP/mAbs structures is incredibly difficult to wade through and needs significant re-writing. By my count, the authors refer to up to 11 reconstructions for 3 conditions: VLP (iso, 2f, and 5f), VLP+C34 (iso, 2f, 3f, and 5f) and VLP+C37 (iso, 2f, 3f, and 5f). However, for this reviewer, it is seldom clear which reconstruction the authors are referring to. Further, the figures that support this section, while visually are generally very nice, do not actually help unpick the results as they are presented. The text itself only refers to figures typically once (in the opening statements) and then never again, which is not helpful.

Thank you for your valuable feedback. We have rewritten the section on structural characterization to improve clarity. As a clarification, we have listed all the solved structures below and welcome any further suggestions from the reviewer regarding their presentation. We have solved the following structures:

1. Cryo-EM structure of the VLP,
2. Cryo-EM structure of the infectious virion,
3. Cryo-EM structure of the VLP-C34 Fab complex,
4. Cryo-EM structure of the VLP-C37 Fab complex,
5. Cryo-EM structure of the C34 Fab-E1-E2 heterodimer complex,
6. X-ray crystal structure of the C37 Fab-E1-E2 heterodimer complex.

For structures 3–4, we performed block-based reconstructions using 2f, 3f, and 5f symmetry axes (and 2f and 5f for structures 1-2) to mitigate the Edwards sphere effect and applied local refinement to improve local resolution. We have summarized the relevant information for these structures in table S1, and the analysis of each structure were presented in detail in subsequent sections.

We hope this revised approach provides a clearer and more structured presentation of the data.

Line 188-189, the authors state that they used locally filtered maps to account for resolution decay in the Fab region. Yet, this does not appear in the methods. This is important detail to include as local filtering, in particular for EM maps.

We conducted local filter by the local filtering option in cryoSPARC. We have updated the details in methods and included a local-resolution map in the supplementary materials.

Line 189-190, the authors state that “fully occupied Fab densities were observed at the 2f, 3f, and 5f vertices…” What do the authors mean by this? That the Fabs are fully there (i.e. fit'able) in all reconstructions? Because this certainly not the case.

We modified this sentence to “On each reconstruction with Fab molecules bound, we could observe densities in all the 80 equivalent binding positions.”

A non-exhaustive list of things for the authors to check in relation to their structural data: -

In the 2f_block_C37VLP structure, long stretches of Fab are missing/not supported by the provided maps. In Chain O, this is from residue 120 onwards. In chain P, this is from residue 110 onwards.

In the model building section, we took into account the equivalent densities in different blocks. For example, the chain O and P in the 2f block of either C34-VLP or C37-VLP structure actually refers to the Fabs around 5f axis. These fabs, especially the CH/CL regions, as I mentioned in the result section, are of lower local resolution and their densities are filtered out when filtering to a uniform resolution by cryoSPARC in sharpening process. If we examine the local filtered map of the 5f block, we can see reliable densities of Fabs in the position of chain O and P, (though with low resolution). Since the CH/CL regions exhibit consistent spatial structures in almost all antibodies, with the main differences arose from the variations in spatial distance and angular changes between these regions and the Fab region, we also built rigid fitted model there. We could upload the local filtered map of 5f vertex for reference if needed.

In the C34_E1E2 structure, volume for residue ~290 onwards in chain B is missing and yet something has been fit.

Same as above question. The model is supported by local filtered map. As well, this fitted model agree well with previous published structure models.

In the X-ray crystal structure, denoted C37_refine_5, there is no believable 2Fo for residue 290 onwards in chain F, and yet something is fit here. Could the authors provide difference maps for this area?

We could upload the 2Fo map for this structure.

The authors state that they used “localized 3D classification” to explore whether broken density was due to partial occupancy or high flexibility. Separately, they indicate processing was conducted in cryoSPARC. However, the reference to ‘T’ values, which are used by Relion leads me to question this. What did they actually do here?

The reconstruction of final map was achieved in cryoSPARC, while for the local no-alignment classification, particles and reference maps were transferred to and ran in Relion. We have updated the method section to detail this part along with more detailed description for block-based reconstruction.

I think also that the question the authors posed is never actually answered. They found a single class incl. 95% of particles, and this showed 5 Fabs bound around the 5f vertex. The authors were also able to fit 5 Fabs bound in this area without direct clashes. So why would 5 Fabs binding at this site in this way cause instability? Maybe it does but it's not clear that there's a structural basis for that conclusion.

Thank you for your insightful comment. Regarding the stability of the Fab binding at the 5f vertex, we prefer to focus on the observed results rather than speculate on the exact structural reasons behind them. The key findings we observed are as follows: the binding angles of C37 Fab and C34 Fab differ, and there is a noticeable discrepancy in their overall occupancy, with C37 showing stronger signals while C34 signals are comparatively weaker.

There are a few possible explanations for these observations. One possibility is that each vertex may only accommodate 1 to 4 C34 Fabs at a time, rather than all 5, resulting in a lower overall occupancy for C34, similar to what was observed with the CHK-263 model. In this case, we would expect to see clear clashes between adjacent Fabs, and the data could potentially be classified into a few distinct classes using a no-alignment 3D classification approach.

Alternatively, the limited space at the 5f vertex may create significant steric hindrance when trying to bind five Fabs simultaneously, preventing several Fabs from binding in random orientations. This could result in a more diffuse density for C34 compared to C37, and would not be readily classified into distinct groups using no-alignment 3D classification. Furthermore, this scenario could involve continuous fluctuations of the Fab constant regions, leading to increased flexibility.

We believe the latter explanation provides a reasonable account for the lower density of C34 Fabs at the 5f vertex compared to C37 Fabs.

The comparison between chains is highly problematic here. The text reads: “The S26 residue of the C34 light chain was in close proximity to the CH domain of a neighbouring Fab.” If you look at the structure, not only is there no convincing volume for the S26 chain at a reasonable rmsd itself but the closest the sidechain is to neighbouring chains is either 12 Å (chain G) or

16 Å (chain Q).

This is important as it is a key argument made for “a weak but widespread crosslinking effect among neighbouring Fabs, potentially enhancing multivalent effects and neutralization potency.” In my view this, conclusion is not backed fully by the data contained within the manuscript.

Please refer to the pdb file named ASU_2f_C34VLP of C34_VLP structure. In that structure, S26 of chain N and N215 of chain S are adjacent to each other with a distance between sidechains of the two residues less than 3 angstrom. Similar crosslinking effects are observed in C37_VLP structure between K189 of chain N and S223 of chain S. However, similar crosslinking effects are not observed in CHK-263 structures, suggesting the crosslinking of trimeric spikes is a potential neutralizing mechanism of C34 and C37 different from CHK-263.

The authors explore the potential for interviriion crosslinking by dynamic light scattering (DLS) finding that the IgG form of C34/C37 induced dramatic increases while the Fab forms did not. In Figure 3m, the authors plot the single condition tested for this, however, for this reviewer, this is insufficient. To really explore the impact of the C34/C37 Fab/IgG on VLP crosslinking, testing a series of molar ratios would be helpful. This would allow the authors to show convincingly that with an increase in Fab/IgG, you see fold-changes in VLP crosslinking.

We performed a supplemental experiment for titrating a series of molar ratio of C34/C37 Fab/IgG on VLP crosslinking and included the results in Fig 3. From the result, we can see the crosslinking effect of both IgGs are “molar ratio dependent” with a slightly stronger crosslinking effect of C34, which aggregate VLPs in a lower molar ratio. CHK-263 IgG and all Fab molecules are not capable of crosslinking different VLPs, which is consistent with previous results and the underlying mechanisms of the neutralizing antibodies.

For this reviewer, the section on C34/C37 blocking MXRA8 attachment very confusing. The authors state that “docking MXRA8 and the corresponding Fabs onto … these three interfaces revealed direct steric hindrance at interfaces…” Except, the figure supporting this assertion shows some protein domains overlapping with a squiggly line.

The authors state that all Fab fragments overlap with the MXRA8 stalk region, but again, I cannot really see this from the figures provided.

I really do not understand 5f and 5g. What are they telling us? The text in the manuscript is just not helpful in this section.

The hypothesis, as far as I understand it, is that the antibodies bind to the spike, and that this then precludes binding of the MXRA8 stalk region to the spike. I am not convinced, however, that this is proven by the data here. To me, it would seem fairly straight forward to test, however, by designing a MXRA8 construct missing the stalk region? Even if this is just in vitro, you could then design an assay to explore the interaction of the spike, MXRA8 (or not, as the case should be), and antibody?

Thank you for your valuable comment regarding the blocking mechanism of the antibodies. Based on a comprehensive analysis of functional assays, structural studies, and cell-based

blocking experiments, we conclude that C34 and C37 are blocking antibodies.

Firstly, functional experiments demonstrated distinct neutralization activity for C34 and C37 in pre- and post-attachment assays, suggesting a potential blocking mechanism. Moreover, MXRA8 has a unique binding mode, in which its 48-aa stalk region plays a crucial role in binding, making it an important structural domain.

Structural analysis revealed that the blocking effect is likely due to steric hindrance with the stalk region. However, because the stalk region is a flexible loop, we could not directly observe it in cryo-EM or crystallography densities. Instead, we inferred its orientation based on the visible residues from the models. In Figure 5b-d, three possible scenarios were proposed for steric hindrance, based on the spatial relationships between MXRA8 and the neighboring E protein on different interfaces. Despite the T=4 symmetry of E proteins on the virus surface, any interface that generates steric hindrance can result in observable blockade of MXRA8 binding.

To further validate this hypothesis, we performed cell-based flow cytometry assays. Compared to in vitro methods such as ELISA, flow cytometry provides a more accurate representation of antibody-mediated receptor blockade in a physiological context.

We conducted flow cytometry experiments in two ways to validate the blocking activity of C34 and C37. In the first approach, we used intact VLPs and let MXRA8 expressed on the cell surface. The results, shown in Fig. S8, confirm that both C34 and C37 are blocking antibodies.

Due to the necessity of the specific sequence within the stalk region for receptor binding and its unique binding mode (potentially influenced by glycosylation sites, as discussed in our 2019 Cell paper), removing or substituting the stalk region in this context does not fully preserve its functionality. To address this, we employed a second approach where we expressed E protein on the cell surface, added antibodies for blocking, and then assessed the impact on MXRA8 receptor binding using different MXRA8 constructs. This is represented in Fig. 5e-g of the earlier manuscript.

In this setup, the C-terminal strep-tag is positioned at the position mimicking cell membrane of infected cells, and we cannot delete the stalk region entirely, as this would bury the strep-tag within the E protein pocket, preventing detection with fluorescent antibodies. However, we could replace the stalk region with an equivalent-length GS linker.

Additionally, to ensure rigorous testing, we further used two complementary configurations by placing the **strep-tag at the N-terminus**, which allowed us to directly assess the effect of both the presence and truncation of the stalk region on receptor binding and antibody blockade. We have updated the experimental setup in the new Fig. 4e and directly show the degree of receptor blockade by C34, C37, and CHK-263 antibodies in Fig. 4f. The results clearly demonstrate that blocking depends on the stalk region, whether the strep-tag is at the N- or C-terminus. Based on these findings, we are confident that the stalk region is the target for the blocking function of these antibodies.

// METHODS //

There is a general lack of information relation to the E1-E2 expression and purification. Into which plasmid was the expression construct cloned, and how? How did the authors harvest the Hi5 cells and into which buffer? How were the cells lysed and supernatant clarified? What buffers were used for the His-tag purification? What buffers were used for the gel filtration step?

The cell was not lysed as protein was expressed secreted. We updated the reference.

The authors tell us that the E1-E2 fusion protein was further 'purified' using an ion-exchange column without providing any information on this. It should be provided. How was the fusion protein concentrated and stored?

The ion-exchange chromatography (Q column) step has been positioned between affinity chromatography and gel-filtration in our purification protocol. We have accordingly revised the methodological description in the relevant section.

In relation to the crystallisation, it is not clear to me how the authors prepared the C37 Fab-CHIKV E protein complex. They reference procuring the complex from gel filtration but the only reference to gel filtration in the methods is in relation to the E1-E2 fusion construct and not a C37 Fab?

Where did the protein come from for the complex, and what was the stoichiometry of the complex? The authors also indicate that the complex was concentrated. Is that correct, the i.e. the reconstituted complex was concentrated or was each component concentrated separately and then mixed together?

The Fab and E1-E2 proteins are first purified separately, and then survived on a superdex 200 column with a molar ratio of roughly 1.5:1 (excessive amount of Fab). We have updated the methods section.

The authors tell us that initial crystals were obtained using "several similar conditions." They should just tell us what those are, providing ranges, if necessary.

"Similar conditions" means the conditions for initial crystal growing conditions with similar salt and pH. We included the detailed crystallization screening kit for clarity.

Can the authors reflect on what "initial crystals" to "improved crystals" actually means? Were they (more convincingly) single? Well-defined? Large? What was it that the authors were seeking to improve?

The optimized crystals are growing larger and diffracted to a higher resolution.

How did the authors determine space group? Which programme? This should be clarified and referenced.

Indexing and data reduction were both done by HKL-2000.

The paper reference for the molecular replacement search model should be included at line 602.

We appreciate the detailed suggestions on methods. We have revised them accordingly. Please see in the revised version of manuscript.

I am not sure what the authors mean by showing “good stereochemical quality” in relation to the Ramachandran plot. There is no agreed target for favoured/allowed/outliers.

We have deleted the quoted words.

I cannot find a Table S1 including data on the crystal structure. This is a major omission. For reference, as far as I can tell, there is only a Table S1, which references exclusively the cryo-EM structures included in the paper.

We appreciate the reviewer to point out this omission. Crystal data details are now included in Table S2.

I have several questions relating to the cryo-EM sample preparation. What were the Vitrobot parameters used during grid preparation? In particular, temperature, humidity, blotting time, and blotting force.

The detailed parameters are now included in methods section.

It is considered good practice, where you have multiple different data collections to split these and report the magnification, defocus range, and total dose for each. This information is currently mixed in the methods.

We actually included this information in table S1 and referenced in method section.

The authors state that particles were picked from micrographs using Topaz. How and on what data did they train Topaz?

References are missing for programmes used in lines 676–685, in particular, MolProbity and Rosetta.

We appreciate the reviewer to point out above deficiencies in text and figure details. We have

updated them in the revised manuscript, except otherwise stated.

// FIGURES //

Fig 1b – this reviewer finds it incredibly hard to work out which curve on the greyscale is what – not accessible at all.

We intended to highlight the C34 and C37 with outstanding neutralization potency. We adjusted the color key of the figure and updated the figure with error bar in a vector-based image, which can be zoomed in to discriminate which line is which.

Figure 2 – for the survival data, it is very difficult to see what data points are statistically significant by comparison to each other. The authors may wish to consider picking 1 or 2 key timepoints, let's say dpi 3 and dpi 15, and then plotting the data only for those timepoints.

For the survival data, we plotted 15 dpi with PBS group. And for body weight and footpad size, we plotted each data point with corresponding PBS group. Data with no significance were automatically left blank with no mark.

Figure 4 – it would be useful for the authors to indicate a database ID for the provided virus sequences.

We have updated the database ID in the figure legends

Figure 5 – I have made several comments in relation to this figure set above, I will not repeat them here.

We are missing any text for the Figure S7 and S8 figure legends.

Figure legends are updated now.

Reviewer #3 (Remarks to the Author):

Brief summary:

Han et al. describe the isolation and characterization of two neutralizing and protective anti-chikungunya virus (CHIKV) human monoclonal antibodies, C34 and C37, from a convalescent patient of CHIKV infection. Through in vitro studies, the authors determine that C34 and C37 inhibit viral entry through aggregation of virus particles and blockade of Mxra8 binding. Structural analyses elucidated the mechanism of C34 and C37 Fab binding to an epitope within β -ribbon cleft on the E2 glycoprotein, a region normally occupied by the E3 protein. However, there are major points that are missing to provide a greater impact to the field. Furthermore, the novelty of the manuscript is moderate as previous studies have shown

similar epitopes and neutralization mechanism(s) of action. To improve the manuscript, the authors should address the corresponding comments.

General concept comments:

The authors define the E2 β -ribbon cleft as a rarely studied epitope. However, several reports describe this epitope and the neutralization mechanism(s) of action similar to those described here (Quiroz et al., 2019 [PMID: 31697791], Zhou et al., 2020 [PMID: 33087569], Raju et al., 2023 [PMID: 37196061]). The authors state that this is an “unique” (line 91) or “novel” (line 339) epitope, however given these previous studies, this epitope does not appear “unique”. Furthermore, several examples of mAbs and their corresponding epitopes are shown in Figure 4F and stated that CHK-263 “binds to an identical epitope” (line 322). This epitope is also not “unique” to CHIKV but to other alphaviruses, such as WEEV (SW24; Sutton et al., 2023 [PMID: 37295404]). Can the authors remove the “unique” aspect of C34 and C37 and provide a description of these previous studies in the introduction? It would also be helpful to include a more detailed discussion comparing C34 and C37 to these previously described mAbs (CHK-263, CHK-152, 516.C10, 516.H09, 506.C07, etc.).

The authors describe in the introduction that “how these antibodies affect CHIKV’s interaction with its receptor remain poorly understood.” However, multiple antibodies have been described that block Mxra8 binding and are not referenced in the proper context of the corresponding manuscript: “Zhang et al., 2018 [PMID: 29769725], Zhou et al., 2020 [PMID: 33087569], Powell et al., 2020 [PMID: 32783883], Raju et al., 2023 [PMID: 37196061].” Can the authors please describe how their studies differ from these previous studies and expand on the “novelty” of the data generated in this manuscript? It would be helpful to compare the Mxra8 blocking activity of C34 and C37 to these previously described mAbs.

Thank you for your insightful comment and for raising a very important point regarding the novelty of our study. We fully acknowledge the existence of previous studies on CHIKV antibodies that exhibit blocking activity or other neutralizing mechanisms, such as Zhou et al., 2020 (PMID: 33087569), where the CHK-124 antibody clearly acts as a blocking antibody. However, the epitope targeted by C34 and C37 differs significantly from CHK-124. In particular, the epitope of C34 and C37 resides at the top of domain B of E2 and the beta-ribbon connecting domain B and domain A. Antibodies targeting this epitope are generally thought to neutralize CHIKV by stabilizing the fusion loop, which inhibits viral fusion or budding, as seen in the CHK-263 structure study.

In contrast, our study presents novel findings. Through structural analysis and flow cytometry experiments, we demonstrate that C34 and C37, which target this epitope, possess an additional receptor-blocking activity. Importantly, we provide the first direct evidence that this blocking function is mediated through interference with the interaction between MXRA8’s stalk region and the E protein. This new insight into the mechanism of action for these

antibodies represents an important advancement in understanding CHIKV neutralization.

Can the authors provide a better description of the E2-E1 fusion protein (EFL) and the E protein? The methods are not very detailed. Does this refer to p62E1, E2-E1 heterodimer, or full-length envelope protein? Usually, the proteins are referred to as the corresponding E2 or E1 proteins, p62E1, E2-E1 heterodimer, or virus-like particle (VLP).

We have updated the expression and construction details in revised version of method. Hope this will clarify the concerns.

The authors observe binding of C34 or C37 to the E2-E1 heterodimer but weak binding to monomeric E2 or E1 protein. However, the authors showed data that supports C37 binding to the CHIKV E2 protein. Please explain this discrepancy. Furthermore, the weaker binding could be explained by a conformational difference in display of the epitope between the heterodimer and monomeric proteins. Please include a discussion about this.

We thank the reviewer for pointing out the discrepancy between the binding of C34 and C37 to the E2-E1 heterodimer versus their weaker binding to the monomeric E2 or E1 proteins. We would like to clarify this point and provide further insight into the data and potential conformational effects involved.

As shown in **Figure 1a** and **Figure S1**, we observed a weak binding of C37 to the monomeric E2 protein, but this binding trend should still be considered relatively weak, especially given the high sensitivity of the flow cytometry detection method. Compared to the binding to the E1-E2 heterodimer, the binding to the E1 or E2 monomer is clearly diminished. This discrepancy is not unexpected, as the structural context of the protein in the monomeric form versus the heterodimeric form can have a significant impact on antibody binding affinity.

In our structural studies, the primary binding epitopes for both C34 and C37 are localized to the E2 protein. This observation might seem to contradict the functional assays, but it can be explained by the unique structural characteristics of the CHIKV E protein. Specifically, the domain B of E2 may only achieve relative stability in the context of the E1-E2 heterodimer, which is a requirement for the full functional activity of the protein. As a result, the conformation of the E2 protein in the heterodimer may be more favorable for antibody binding, while the monomeric E2 may exhibit a less stable or altered conformation that is less accessible for binding by these antibodies.

Thus, the weaker binding observed with the monomeric proteins could indeed be due to a conformational difference in the display of the epitope, as suggested by the reviewer. This conformational change could lead to a reduced accessibility or a less stable presentation of the epitopes for C34 and C37 when E2 is in its monomeric form. This explanation is consistent with the observed functional results and underscores the importance of the E1-E2 heterodimerization for the proper presentation of the binding sites for these antibodies.

We hope this additional clarification resolves the perceived discrepancy and provides a more comprehensive understanding of the observed binding behavior.

We include the following paragraph in the relative section: "It is worth noting that although

C37 shows weak binding to the E2 protein, its binding ability to the E1-E2 heterodimer is significantly stronger. This may be due to the more stable conformation of the E1-E2 heterodimer, which could result in differences in the epitopes between the E2 monomer and the E1-E2 dimer."

The flow cytometric cell sorting data of CHIKV-reactive IgG⁺ B cells are missing. Please include the data in the manuscript.

Thank you for your suggestion. Our primary goal was to identify high-affinity neutralizing antibodies from the sorted B cell library, rather than to conduct a comprehensive study of the antibody repertoire following infection. As a result, we did not retain the raw data from the initial B cell sorting.

The authors state that "blocking cell attachment is a **key** mechanism of neutralization for C34 and C37" (lines 138-139). However, the relatively strong neutralization activity of C34 and C37 in the post-attachment assay indicates that C34 and C37 do not prevent virus attachment as a main neutralization mechanism of action. This may in part contribute as a mechanism of action. However, given the modest neutralization activity of C34 and C37 this indicates otherwise. Please modify the conclusion appropriately.

We conducted further functional experiments including Egress inhibition and fusion inhibition experiments. Results and conclusions are modified accordingly.

The described "triple-action" mode for viral entry inhibition has been described before. To further define the neutralization mechanism(s) of C34 and C37, additional studies are needed. For example, do C34 and C37 inhibit viral fusion, egress, or cell-to-cell spread? Please perform these experiments as they are readily established in the field.

We conducted the egress inhibition experiment and FFWO experiment to test the fusion inhibition to certificate their role in neutralization. We have modified the above paragraph associating with pre- and post-attachment experiments into the following ones:

"This suggests that although blocking cell attachment plays a role in the neutralization mechanism of both C34 and C37, they still exhibit strong neutralizing activity after attachment, indicating that inhibition of membrane fusion and budding may also contribute to their overall neutralizing effect. We next conduct FFWO and egress inhibition experiments."

The results are included in Fig. 1. We included the results of above experiments in main text.

The relatively strong neutralization activity of C34, C37 and other antibodies binding to this similar epitope were historically believed to neutralize through inhibiting fusion and budding. However, in our study, we have identified additional mechanisms by which C34 and C37 exert their neutralization effects. Specifically, we found that these antibodies can block virus-

receptor binding by sterically interacting with the stalk region of the virus. Furthermore, in their IgG form, C34 and C37 exhibit cross-linking activity between viral particles, as well as between virus particles and host cells.

These combined mechanisms—blocking virus attachment, inhibiting fusion and budding, and cross-linking—together form a triple action mode of neutralization, which represents a key finding of our study. We have modified the conclusion in the manuscript to reflect this more comprehensive understanding of the neutralization mechanisms.

The *in vivo* studies are not described very well. Please expand on these studies. Why were mice aged 8-12 weeks old chosen (usually 4-5-week-old mice)? Footpad injections in immunocompromised models are generally lower viral titers (10¹ to 10³ FFU). What strain of CHIKV was used? Were the mice challenged by footpad or subcutaneous injection? These are used interchangeably. Please keep consistent as “subcutaneous inoculation of the footpad”. Was a negative control mAb (instead of PBS) included for comparison? Did the authors correct for multiple comparisons in statistical analyses?

We would like to provide further details to address the points raised and clarify the experimental design.

Regarding the choice of 8-12-week-old mice, we selected this age group primarily to minimize the potential for non-lethal challenges. Older mice may exhibit a lower susceptibility to viral infection, potentially confounding the interpretation of the protective effects of the antibodies. Through preliminary experiments, we determined an appropriate challenge dose of 100 times the LD₅₀, which is a relatively high dose that ensures robust viral infection and allows us to assess antibody efficacy under conditions where protection can be clearly demonstrated. Additionally, we specifically used 8-12-week-old female mice in both our pre-experiment and formal experiment, and the high challenge dose was sufficient to rule out any concerns related to age-dependent differences in viral susceptibility. Our results showed that mice in the PBS control group succumbed to the infection within 5 days post-challenge, while those treated with protective antibodies were fully protected from death, which further supports the validity of the protection observed in the 8-12-week-old mice.

Regarding the strain of CHIKV used, we employed the 181/25 strain, a well-characterized attenuated strain. This strain was chosen based on previous studies, and we confirmed its suitability for our experimental design through preliminary testing. The mice were challenged via subcutaneous inoculation on the footpad, as this method ensures consistent infection and allows for precise measurement of viral load in local tissues. We have now ensured that this terminology is consistently used throughout the manuscript.

To address the reviewer's concern about the inclusion of a negative control antibody, we did include an additional non-protective antibody, C59, as controls. This antibody did not provide protection, and the mice receiving C59 gradually succumbed to infection starting from day 5 post-challenge. The corresponding data for control group is shown in the modified Fig. 2.

The authors suggest that full occupancy is achieved in the observed reconstruction. However,

this is an average of particles. Can the authors please expand on why full occupancy would be observed? Furthermore, as there are residues within proximity of each other (lines 211-213) and the authors describe a potential “cross-linking effect among neighboring Fabs” (line 215), would an IgG be able to bind due to the presence of the Fc region? Please include a section about this in the results and discussion.

For the “full occupancy”, We conducted the block-based local classification and refinement for the particle stacks. Shortly say, in the VLP with T=4 icosahedral symmetry, there are 4 identical E proteins in an asymmetry unit, as we have built the model in the 2f block reconstructions. If densities of Fab molecules on that reconstructed map are solid enough, then the occupancy is “full”. We also conducted no-alignment 3D classification. If only partial occupancy of Fab is achieved, there would be a subset of particles to reconstruct a volume with no Fab densities, which is not the case in this study. So, the occupancy is full in our case.

Whether IgG will be able to bind due to the presence of Fc region, it is related to the distance between adjacent Fab molecules. We plotted this distance in Fig.3 g, k, h and l. A distance shorter than 50 angstroms usually indicates a possibility of simultaneous binding within one IgG molecule, otherwise, it is too far to be linked by the same Fc molecule, such as the case in Fig. 3g, where the distance is longer than 50 angstroms.

The authors observed distances <50 Å between neighboring Fab molecules of C34 and C37, which enables intra-virion cross-linking as previously described for EEEV Fab molecules. However, when assessing for aggregation of VLPs by dynamic light scattering, the authors observed significant aggregation, which suggests inter-virion cross-linking. However, these observations counteract each other. At what VLP:IgG molar ratio was the DLS experiment performed? If the authors titrate the molar ratio, are there differences in the aggregation potential of C34 and C37?

The term intra-virion cross-linking refers to the possibility that two adjacent or spatially close Fabs on the same virion might originate from the same IgG molecule, while inter-virion cross-linking refers to the potential for two Fabs on the same IgG molecule to bind to different virions, thereby clustering them together. These are two distinct yet non-conflicting modes of binding. Both could occur simultaneously, neither may occur, or only one might be present at a time.

The degree of aggregation observed by dynamic light scattering (DLS) reflects the extent of inter-virion cross-linking, as antibodies can cause the aggregation of multiple virions, leading to a significant change in the particle size distribution. In contrast, if only intra-virion cross-linking occurs, no substantial changes in the DLS results would be expected because each virion remains independent, with only additional antibody molecules binding to them.

We have also included DLS data under different antibody-to-virus molar ratios, which better illustrate the aggregation of virions induced by inter-virion cross-linking mediated by C34

and C37 antibodies. The relevant results are presented in the revised Figure 3.

The authors describe residues N245 and D250 (line 269) as pivotal for C34 and C37 Fab molecule binding. How did the authors come to this conclusion? Mutagenesis or viral escape studies are warranted to understand the contribution of these residues for Fab/IgG binding and neutralization.

We reached this conclusion for two reasons: First, through conservation analysis, we found that these two antibodies do not cross-bind to the E proteins of many other alphaviruses, suggesting that the non-conserved residues may be involved in the interaction. Second, structural analysis and comparison revealed that the side chains of residues D250 and K252 adopt different orientations in the presence of E3 binding, VLP, and antibody-bound VLP, and are within a reasonable range to form hydrogen bond interactions. Based on these observations, we concluded that these residues play a key role.

Due to the complexity of constructing mutant viruses, we instead validated our hypothesis using cell-based mutation experiments. Given the large interaction surface of these antibodies with the E protein and the high-resolution structures we have obtained, we believe there is no particular need to validate every individual residue pair at the interaction interface. We therefore speculated that classic point mutation biochemical assays might not fully eliminate their interaction. However, to ensure rigor, we still performed flow cytometry to assess the impact of key residue mutations on affinity. The results, shown in the supplementary figure, indicate that the K252D mutation had the most significant effect. This residue is located at the loop conformation change between residues 245-250, which supports the validity of our original conclusion.

In this manuscript, the authors state that a reconstruction of infectious CHIKV particles was generated. However, there is no reference to these data in the manuscript. Can the authors please include these data? Furthermore, PDB ID: 6NK7 was used to build an atomic model (line 289). However, this also contains the mouse Mxra8 receptor, which may yield an inappropriate initial model to build upon.

The overall structure description is now included in the Overall structure section.

We manually made point mutations on every amino acid from the beginning of model building to ensure the sequence identical to human Mxra8 receptor. The reason for this choice is that this structure was the only one with a full-length structure of VLP at the timepoint when the structure model was built.

The authors describe that C34 and C37 “inhibit the exposure of the fusion loop” (line 344). Can the authors please expand on how they came to this conclusion? It would be helpful to include studies assessing fusion inhibition.

We have updated the FFWO experiment and included in Fig.1. C34 and C37 both show

considerable level of inhibition of the fusion loop.

In the discussion, the authors state that the mAbs isolated from the CHIKV patient have different mechanism(s) of action compared to those elicited by CHIKV VLP vaccination. However, Raju et al., 2023 [PMID: 37196061] isolated and characterized mAbs that recognize similar epitopes, potentially neutralize, and protect against CHIKV infection. Can the authors please provide a more direct comparison to this publication? Furthermore, this is a strong statement with little evidence to support this and requires additional studies.

The antibody from PMID 37196061, including 506.A08, 506.C01 are binding to an essentially different epitope other than C34 and C37. One major different is binding epitope of that two antibodies do not include K252. From a direct structural alignment of those two antibodies with C34 and C37 structure could also lead to an apparent difference of angle of engagement. The epitope of 506.A08 and 506.C01 are more conserved among various alphavirus. We will include the epitope comparison of these antibodies in Fig 4f, as well, we will include a direct alignment of structures in the supplemental figures.

The methods could provide more detail. Please include additional information, including strains used, assay details, etc.

We have included more detailed information in method. Database ID for strains are included in figure legends.

Specific comments:

In the results, the first referenced figure legend corresponds to a supplemental figure (Figure S1). The figures should be referenced in order, starting with Figure 1. Please change accordingly.

We have reordered the corresponding paragraph to state the data associated with Fig. 1a first with supplemental figs following.

The authors refer to "ONNV E protein as having the highest sequence similarity with CHIKV-E" (lines 121-122). However, as referenced in Figure 1a or S2 or anywhere else within the manuscript, the analysis is not provided. Figure 4F provides an alignment of the E2 epitope. However, the level of conservation is unclear. Can the authors please provide a sequence similarity analysis of the corresponding viruses and proteins?

We updated the sequence distance in Fig S1. Including some strains of CHIKV and other typical alphaviruses.

Figure 1: Were positive and negative control mAbs included? Please show if so.

For binding experiments with ELISA, we included an isotype IgG as negative control. When

doing cell sorting, we conducted the experiments in batches and control mabs were not included. However, all the positive binding mabs are further verified by SPR (data shown in Fig S3.) and are generally in good accordance with flow cytometry results.

Figure 1B: Please show the error bars.

We have updated the figs and corresponding IC50 values with error bar.

Figure 1D-1E: Please keep the axes consistent for better comparison of mAb activity.

We have adjusted the axes accordingly.

Figure 2 Legend Title: Remove “Pre- and post-attachment assays and”.

We have removed the contents.

Figure 2C and 2G: Please keep the axes consistent.

Figure 2D: Please correct y-axis label.

Figure S6: Please include the FSC for VLP alone reconstructions.

We have corrected the above contents and updated the figure S6 with FSC and local resolution map included.

Figure 3: It would be useful to include a RIVEM image of the relative binding epitope for C34 and C37.

The epitopes of C34 and C37 overlapped in a high degree. As the resolution of our structures are relative high, we identified the exact binding residues and plotted them in Fig 4a-c, we earnestly hope that we are allowed to omit the RIVEM images for conciseness and to avoid redundancy.

Figure 4A-4B: Please change the color of E2 β -ribbon region. It is hard to distinguish between the C34 Fab molecule and this domain.

Thank you for the suggestion. We have updated color in Fig 4.

Title: Chikungunya virus should be all lowercase.

The title is adjusted accordingly.

Line 24: Please change to “illness” instead of “infections”.

We appreciate this suggestion. Modification is achieved.

Line 36: Can the authors explain what they mean by the clash between the antibodies and the stalk region of Mxra8? What is the “stalk region” referring to?

The stalk region refers to a 48 a.a. linker at C-terminal of Mxra8, downstream of the D1 and D2 domain. We have found its essential role in binding of CHIKV virus. Please find more details in our publication in 2019 (PMID: 31080063). The clash between antibodies and the stalk region of MXRA8 means on the intact surface of a virion particle. MXRA8 and C34 or C37 Fab cannot bind simultaneously because there will be steric hinderance between the stalk region with Fab molecule binding to the nearby E protein.

Lines 38-39: Please remove “as a common and pivotal target for antibodies in the same class as C34 and C37” and add “... cryo-EM reconstructions with C34 and C37 identify ...”

Contents are modified accordingly.

Line 41: Please remove “functional and structural” to improve conciseness

Line 55: Please change “E3-E2” to “p62” for consistency.

Line 56: Please keep the proteins in order of the polyprotein reading frame. Change “C, E1, p62, and 6K” to “C, p62, 6K, E1” and “E2 and E3” to “E3 and E2”.

For above 3 suggestions: all contents are modified according to reviewer's suggestion.

Lines 62-66: Discuss the role the E2 protein plays in preventing premature exposure of the fusion loop.

The following sentences are included in introduction: “The E2 protein prevents the premature exposure of the fusion loop, ensuring that the virus remains non-infectious until it encounters an acidic environment, such as that in the endosome, which triggers the conformational changes required for fusion.”

Line 63: Please remove “E2-“ for conciseness. Define DA, DB, DC.

Line 65: Please remove “domains” if the authors want to use DA and DB for conciseness.

Line 68: Please change “fusion peptide” to “fusion loop”.

For above 3 suggestions: all contents are modified according to reviewer's suggestion.

Lines 124-125: Please include the data not shown for Figure S2.

Based on the materials available at the time, we performed experiments using different methods, including flow cytometry and ELISA. For the sake of rigor, we felt it was not appropriate to present these results together. Since this result is not one of the main conclusions of the manuscript, we have decided to remove this section of the description.

Line 127: “In vitro” should all be lowercase.

Line 146: “In vivo” should all be lowercase.

Already corrected.

Lines 143-144: Please expand on the following statement, “this phenotype indicates that the attachment-inhibiting activities are strongly associated with the Fab regions of the two mAbs”.

We have conducted more functional experiments and the conclusions are rewritten for that section.

Line 181: Add “... , respectively, ...” before “... for C34 Fab bound ...”.

Lines 182-183: Were reconstructions of the 3f blocks for VLP alone determined?

For simplicity, we didn’t conduct the 3f local reconstruction for VLP alone because all the E proteins in 3f vertex are essentially included in the 2f and 5f reconstructions. As no extra densities of interest such as antibodies or receptors on the 3f vertex for the VLP alone structure, we think it can hardly present more information of interest by local refinement of 3f vertex.

Lines 184-185: What is the RMSD value upon C34 and C37 binding?

In the T=4 icosahedral symmetry of the viral particle, each asymmetric unit contains four E1-E2 heterodimers, which exhibit slight conformational differences. Upon C34 or C37 antibody binding, we analyzed the structural differences between these four heterodimers and their corresponding counterparts in the antibody-free VLP.

To quantify these differences, we performed pairwise alignment of the corresponding E protein heterodimers in PyMOL and calculated the RMSD values to assess conformational variation.

Line 221: Please describe which cysteine residue was used to measure distances between Co atoms. Cys216?

We used the heavy chain C224 for the measurement, the upstream and downstream sequences are: VEPKSCDKTH

Lines 370-371: Please avoid use of the word “synergy” but instead rely on “cooperative”, since synergy requires more precise definition.

Line 391: Please change “pr” to “prM”.

Please change “MXRA8” to “Mxra8”, unless the authors are discussing the gene. There are many instances of this, but as examples, lines 35, 36, 71, 92, etc.

Please change “Fabs” to “Fab molecules”. There are many instances of this, but as examples, lines 171, 175, 184, etc.

We have modified the representations accordingly.

In particular, the revised manuscript will need to include additional experiments to unequivocally demonstrate that the characterized antibodies block Mxra8 binding. Please ensure that the revised manuscript addresses these and all other concerns raised by our reviewers. In addition, please include the direct comparison to 506.A08 and 506.C01, which you mentioned in the response to reviewers but didn't include in the manuscript files (unless I missed it).

We appreciate your suggestions in addressing the blockage of Mxra8 binding. We conducted additional titration experiment following reviewers' suggestion and updated the results in corresponding figures. We also addressed all the remaining questions the reviewers raised.

The epitope alignments of 506.A08 and 506.C01 are now included in Fig. 4f.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The responses to my review were adequate thank you.

We appreciate your professional and constructive comments and suggestions provided during the review process.

Reviewer #2 (Remarks to the Author):

// Summary //

One of my major criticisms was that the paper was very difficult to follow in several areas. In particular, the structural biology elements of the paper. I think this has improved but there are still areas where I get quite lost.

The findings remain the same in so far that specific monoclonal antibodies apparently have a 'triple' mode of action targeting the interaction of CHIKV with its putative receptor Mxra8. I remain skeptical however, that the blocking/specific interaction with Mxra8 is demonstrated by the data presented in the manuscript, and I explore this below too.

We have included more functional data to support the claim that the two antibodies in our study and previous publications (CHK-263) by other groups are blocking antibodies mainly targeting the stalk region. We also updated the detailed methods.

In my initial review, I said that I thought the authors could do more to highlight what exactly is novel in this work when compared to other recent papers, like Zhou et al. 2020 (PNAS). Other reviewers also highlighted this as a concern.

While I acknowledge and appreciate that the results of the paper has seen significant re-writing, the discussion of the manuscript remains untouched and so the novelty question remains. There is a lot of data in this manuscript. The authors really must reflect on how their studies different from previous studies and expand on the novelty of the data presented here and, importantly, include those reflections in the manuscript itself.

Thank you for your suggestion on highlighting the novelty of our manuscript. We found that although C34 and C37 hold an epitope partially overlapping with CHK-263, the phenotype and neutralizing mechanisms may different from each other. For the phenotype, CHK-263 could not induce aggregation of virion particles while C34 and C37 could. The post-attachment potency of Fab forms of C34 and C37 were reduced less than 10 fold comparing to IgG counterparts, while for CHK-263, the reduction was over 1000 fold, which were common among domain B antibodies. These differences may imply different neutralizing mechanisms of the two types of antibodies even with similar epitopes.

We also conducted the Mxra8 blocking assay in a dose dependent manner and confirmed the conclusion previously drawn. From both the direct superposition and blocking assay, we can better conclude that C34 and C37, probably also CHK-263 do clash with the stalk region of Mxra8 without a direct overlapping between antibody epitopes and the binding site of Mxra8. This observation are in accordance with the potency difference between pre- and post-attachment neutralizing assay.

To better highlight the novelty of our research in the manuscript, we included the following paragraphs in results section: "We also observed that the IgG form of C34 and C37 behaved differently as CHK-263 did in inducing the aggregation of VLPs. The reason is that C34 and C37 bind to VLP with heavy chain adjacent to virion surface, leaving C terminal tails of CH pointing outwards, while in CHK-263, the heavy chain is positioned oppositely and pointing downwards."

We also highlighted the novelty in discussion as follows: "Our structural analysis revealed that C34 and C37 are two antibodies targeting an overlapping epitope positioned on E2 domain B and β -ribbon connecting domain A and domain B. On alphaviruses, E2 domain B is located at the most membrane-distal region, and have been demonstrated as a main target of neutralizing antibodies. Most E2 domain B targeting antibodies are involved in interfering with multiple stages of viral infection, which is also the case for C34 and C37. Two epitopes spanning residues 180-199 and 209-223 are common among E2 domain B targeting antibodies, like CHK-265, 506.A08 and 506.C01. A less characterized epitope spanning residues 245-252 was also covered by some antibodies against various alphaviruses like CHK-26354 against CHIKV, SKW2455 against WEEV, and in this study, C34 and C37. 5455

Among the antibodies with existing structural analyses, the highest epitope overlap with human antibodies C34 and C37 is observed with the murine antibody CHK-263. Based on a 4-5 Å medium-resolution structure, it is inferred that the neutralization mechanism of CHK-263 is inhibition of fusion by locking E1 and E2. Despite the significant epitope overlap between C34, C37, and CHK-263, structural and functional assays reveal at least two key

differences: (1) CHK-263's binding is heavily dependent on the light chain, which is located on the virus membrane-facing side, whereas for C34 and C37, the heavy chain faces the membrane, with the light chain at the far end. A significant outcome of this is that CHK-263 cannot mediate cross-linking of different viral particles through bivalent effects, while C34 and C37 can, as their C-terminal end of CH region points towards the solvent region, facilitating viral particle cross-linking. This finding is consistent with results of DLS (Fig. 3o). (2) The post-attachment activity of C34 and C37 in their Fab form declines less dramatically compared to their IgG form, unlike most domain B-targeting antibodies, including CHK-263. This suggests that antibodies with similar binding epitopes may exhibit distinct neutralization mechanisms. Further investigation into the mechanisms of C34 and C37 reveals that these antibodies block receptor binding by forming spatial hindrance with the stalk region of the receptor Mxra8, which aligns with the phenotypic observation that these antibodies act during the attachment phase in functional studies."

Besides, at least to my knowledge, we determined the structure of CHIKV virion in a highest resolution so far, especially for determining the structure of E3. As this is beyond the scope of this manuscript discussing antibody neutralizing mechanism, we didn't dive too deep into this, but just highlighted the novelty in discussion as "In addition to elucidating the mechanisms of these two mAbs, we generated high-resolution reconstructions for CHIKV VLPs and virions with E3 attached, providing a more precise model of CHIKV particles. These high-resolution models, along with models of various alphaviruses, can guide further structural studies to identify critical determinants of antigenicity and inform the rational design of vaccines against alphaviruses." We think the readers could have their own discretion on this structure if they are interested.

As before, I make further comments in relation to the science in the paper in the following sections. I hope that these comments are helpful.

// Structural Biology //

Line 200. Can I suggest to the authors that they include somewhere a simplified diagrammatic representation of the virus capsid proteins? If you are unfamiliar with the various components, it is very easy to get lost in Figure 3. What is E1 vs. E2, for example? I realise this is explained in the introduction but seeing this makeup would make things easier to understand.

We have included an illustration of infectious virion structure colored by various components in Fig S7. Hope this could help illustrating the architecture of E spike.

Line 215–219. I still find all the different resolutions confusing. Can you put these into a small table? This would also simplify the text greatly… 'An overview of reconstructions and their calculated overall resolutions is provided in Table X.'

We actually have included them in Suppl. Table 1. We modified the interpretation further to

exclude the details in main text. How the different blocks (with different resolution due to various subparticles, masks and volumes) were used to generate the final structural model is further stated in methods.

Line 228-232. The authors tell us that the binding of the C34 and C37 fabs do not alter overall virus assembly. This would be a fairly straightforward finding to show in a supplementary figure showing the overall diameters unchanged between the VLP alone and VLP/Fab reconstructions.

We have included a direct comparison for the diameters in Fig S8.

Line 233. I think the authors mean “asymmetric unit” rather than each “asymmetry unit.”

Thank you for pointing this out, the typos are modified.

Line 260-262. ‘The S26 residue of the C34 light chain was in close proximity to the CH domain of a neighboring Fab, while the R218 residue of the C37 heavy chain was near the Q156 residue of a neighboring Fab’s light chain (Fig. 3m).’ You cannot see this in the figure as it is currently drawn.

The interfaces are circled out and the surface representation of Fab molecules are also included. Readers can see the “overlapping” of the surface representations, indicating a distance less than 3.5 angstrom between indicated residues. We added an arrow pointing to the two interface residues in revision.

Line 274-275. ‘For C37 Fab molecules around the 2f vertex, all measured distances were within the 50 Å theoretical radius for intravirion crosslinking (Fig. 3g-h, k-l).’ I still find this very confusing. Which figure should I be looking at? The figure legend doesn’t help me decode what is going on in Figure 3. If I look at Figure 3g, then the distances are all greater than this theoretical limit?

Fig 3g is for C34, and Fig 3k is for C37. We have updated the figure legends. Hope this will make it clearer.

Line 266-294. Can I suggest to the authors that they sequester this part of the text in its own little sub-section? It reads well and is reasonably self-contained.

Thank you for your suggestion. We put this section under a section titled “Inter- and intra-virion crosslinking effects may contribute to neutralization potency of C34 and C37.”

Line 200-265. I still find this overview complicated and confusing. I think the authors have made improvements, which I greatly appreciate. But I still tend to think that it’s perhaps overly

verbose and makes limited use of figures. As far as I can tell there is a single reference to a supplementary figure?

In my view, the manuscript could use reading by perhaps a collaborator unfamiliar with the experiments to really address some of the difficulties with language.

We have included more figures in supplemental data now according to your suggestions above, to make it less dependent on verbose.

Line 348. 'After block-based reconstruction,' is an incomplete sentence.

The description of the infectious virion structure is rewritten and repositioned in the beginning of this section. This sentence is deleted.

Line 346-369. This part is quite nice to read through but again, there is a lot happening and the authors describe fairly dynamic processes. The only figure referenced is Figure 4e and I am not really sure what that tells me. Can the authors think about ways in which they can better show the dynamism, for example, of the D250 sidechain and its impact on neighbouring residues?

Line 398. What do the authors mean by 'global domains'?

This sentence is rewritten. For clarity and consistency with figure legends, We use "...a 48-a.a.-long stalk region protruding from C-terminal of the D1 Ig domain..." in the modified paragraph.

In response to my comments indicating that various bits of protein are missing from the provided maps, the authors indicate that they used locally-filtered maps to fit these parts of the protein. The authors should make this absolutely clear in the manuscript, specifically what experimental maps have been used to produce the deposited 3D structure.

Thank you for pointing this out. We have updated the model building procedure in detail in the methods section.

The authors offer to upload a 2Fo map for the X-ray crystal structure, this would be helpful to see.

We noticed that on residues 290 onwards, densities are broken under $\sigma=1.0$, even though the Rfree and Rwork are not significantly affected. We therefore removed these residues and refined the structure again against the densities. The deposited PDB is therefore updated. We have uploaded the new pdb and mtz files to Figshare.

Line 371-425. I remain unconvinced at this stage that the title of this section that 'C34 and C37 block the Mxra8 attachment by interfering with the stalk region' is clearly demonstrated by the data shown. See specific comments below.

Figure 5b-d remains confusing to me and, I suspect, others. I understand what it is meant to show, the outcome of protein-protein docking experiments. However, I still do not understand why the authors cannot simply show these experiments? If the authors wish to rely on in silico docking/prediction then they surely have to show this data. For readers, it will be important to see where and how the Mxra8 stalk region sits within the spike. Currently, this is represented by an orange squiggly line, which is not the same thing. Please show the outputs from your docking experiments. Further, as far as I can tell, there is little reference to docking experiments in the methods?

We were actually referring to “alignment” or “superposition” of the antibody to an Mxra8-bound CHIKV E structure rather than an “experiment”. We didn’t do MD simulation as the overall system is so large that only coarse-grained simulation is applicable. However, meaningful conclusion can hardly be drawn from a coarse-grained simulation in this case. Same thing applies for the in silico prediction.

For a better illustration of this “overlapping” or “steric hinderance”, we therefore replaced the figures 5b-c with a direct alignment of C34 or C37 fabs with mouse Mxra8 bound structures as the first nine residues of the stalk region is modelled in that structure (PDB code: 6nk6). As the sidechains were not modelled in the CHK-263 structure due to its moderate resolution, we removed the alignment results of CHK-263 mainchain with the Mxra8 bound structure because it cannot offer conclusive results in an unambiguous way. Nevertheless, in the blocking experiment followed, we still included CHK-263 to examine whether it also have blocking activities as it shares an epitope partially overlapping with that of C34 and C37.

The basic assumption is that the hydrophobic sidechain and abundant glycation position in stalk region may contribute to the blocking activity.

After all, the blocking activities originate from the binding mode. So, we believe a static structure alignment is enough for the structural biology part in this section. We also did functional experiments to further support this mechanism raised by structural analysis, which should be more convictive than an *in silico* prediction.

We modified the figure legends accordingly and removed the red asterisk and triangle indications. Only apparent clashes between C34 or C37 and the fitted stalk region is indicated. For C37 in interface 1, no apparent clash is observed, so we indicated this as “no apparent clash”.

Importantly, we don't come to the conclusion of blocking solely from the structural analysis. Instead, we prudently performed the cell-based receptor blocking tests to further verify this hypothesis, especially acknowledging that the 2020 PNAS paper have left this question open. We included such data in Fig 5 and have updated the results and methods in detail so that the verification of blocking mechanism should be more feasible.

Line 378-381. The authors tell us that docking experiments showed no direct steric hindrance between C34 or C37 and the Mxra8 within a given stalk. But we cannot see this in the figure.

Line 381-383. The authors tell us that there may be “potential clashes” between the Fabs and Mxra8 bound to adjacent spikes. Again, we cannot see this in the figure. The authors should also clarify what they mean by “potential clashes”. What does this mean in practice (i.e. lengths, distances, etc.)? This detail is important particularly if this is the only mode of inhibition (remembering that we have just been told that within a spike there is no steric hindrance).

Line 383-385. The authors tell us that they analysed “three distinct interfaces” when in Figure 5b-d there are 4 interfaces reported? Can the authors show, specifically and unambiguously what interfaces 1, 2, 3, and 4 refer to somewhere? So that this is absolutely clear to readers.

Line 387-390. The authors tell us ‘Docking Mxra8 and the corresponding Fab molecules onto these three interfaces revealed direct steric hindrance at interfaces 2, 3, and 4 for the C34 Fab, while no apparent clash was observed for other docked interfaces (Fig. 5b-d).’ However, we cannot actually see this in practice in the figure. See comments above.

Line 389-390. I take specific interest in this comment ‘... no apparent clash was observed for other docked interfaces.’ I am not sure that is true based on the figure legend for Figure 5. The figure legend tells us that a red asterix indicates an “apparent clash”, a red triangle indicates a “clash” and red circle indicates a “potential clash”. So based on this categorisation, actually there are clashes reported for interface 1 (C34), and interfaces 2, 3, and 4 (C37). Potential clashes exist in other cases.

In regard of the “three distinct interfaces”: In figure 5a, we plotted the 4 interfaces within one single asymmetric unit or with adjacent asymmetric units. To note, all over the surface of CHIKV VLP particles, there exists only 4 equivalent interfaces in a T=4 symmetry. Shortly saying, the two i3 spikes lies exactly parallel to each other, so that the interface 3 and 4 are also identical when discussing the interactions between antibodies and receptor. The interface between q3 and i3 spikes, however, is not perfectly parallel. So that at interface 1 (receptor on spike B, antibody on spike A), the receptor is a little bit far away from the antibody comparing to receptor 3 or 4. Similarly, at interface 2 (receptor on spike E, antibody on receptor A), this distance is shorter than that in interface 3 or 4. As this definition is true across all the alphaviruses and has been illustrated for multiple times in various publications (especially the ones reporting receptor structures), There is no new knowledge to the field. We thus put this diagram here to show which interface we are referring to in the following sections and won't discuss this in depth.

For the observation of direct clashes. We have updated Fig5.b-c with alignments of antibodies to mouse mxra8. We only depict the “apparent clashes” and “no apparent clash” in the updated figures to make it more straightforward.

// Methods //

I thank the authors for now including the classic “Table 1” for their X-ray crystal structure. However, I note several discrepancies between the table and the manuscript text.

For example, the data collection wavelength is different (0.97915 in the paper vs. 0.97853 in the table). Similarly, the final Rwork and Rfree values are different (for Rwork, the paper has 0.239 whereas the table has 0.2216). The Ramachandran values are also different. For example, 0.43% of residues are outliers in the paper, 0% are outliers in the table.

Thank you for pointing this critical mismatch out. We double checked our results and found that the parameters in suppl. Table are correct. The wavelength and detector type in the method section was mixed up with a set of data collected in BL17U which was not used for figure production. The Rfree, Rwork and Ramachandran value was filled in with the initial round of auto-refinement by phenix in the initial stage of the draft preparation and failed to get timely update after following manual and auto refine in Coot and Phenix. The values in last version of Suppl Table2 were final and match with the PDB report. Plus, according to reviewers' suggestions, we have removed the 290 a.a. onward of E1 in the C37-E1-E2 complex structure so that pdb model fits more rational with the density. The Suppl Table 2 is thus updated again accordingly. We have also updated the methods section.

I suggest the authors double-check the figures in the paper and table in relation to their X-ray crystallography experiments.

In my initial review, I left this comment: "It is considered good practice, where you have multiple different data collections to split these and report the magnification, defocus range, and total dose for each. This information is currently mixed in the methods." To be clear, by this I meant... where you have multiple data collections (with different parameters, though, not always) pooled into a single dataset. Does this apply here?

This is reported in the Suppl. Table 1, separately for each dataset. You can also see, we do have multiple data collections. However, for any single sample, we never merged dataset from multiple collections for constructing one structure.

// Figures and Tables //

I think the two supplementary tables are missing legends. These should be included allowing the authors to clarify how key metrics were calculated, for example, Rwork in the case of their X-ray crystallography experiments.

Supplementary Table 1. The map resolution range for all reconstructions is missing. Ditto for model resolution range, although, I am not sure how these would really be different here?

These two metrics are the same, so we would only keep the ones in the block-based refinement section. The resolution range refers to the resolution range from local resolution estimation job from Cryosparc.

Reviewer #4 (Remarks to the Author):

I've been asked to comment on the revisions performed to Reviewer #3's concerns.

The authors have adequately addressed many of the points raised by Reviewer #3, however, some issues require clarification.

Key Remaining Issues:

1. The main point of novelty in this manuscript is the claim that C34 and C37 block MXRA8 engagement by blocking the stalk. However, the data supporting this claim remain problematic. It is clear neutralizing mAbs do not share overlapping epitopes with MXRA8, and blocking effects seen in other studies may simply be due to non-specific steric blockade.

a. A negative control mAb which does not “block the stalk” would help validate the specificity of the assay.

b. It is clear between Stalk (N-tag) and no stalk (N-tag) that the MFI of no stalk is higher possibly explaining diminished “blocking” activity observed with the mAbs. The same is true between Stalk C-tag and GS C-tag (1 log higher MFI). These should be titrated so that there is similar staining in the control so blocking activity can be more directly compared.

c. The data may be more clear by titration of the blocking mAb as well.

d. Quantification of the assay by MFI is necessary along with appropriate statistics.

e. The section in the results describing the experiments performed are quite difficult to parse through.

Thank you for the constructive suggestion. We included a published mAb SKT20 as a negative control. SKT20 is a weakly neutralizing antibody reported to bind the fusion loop of CHIKV-E and not supposed to interfere with Mxra8. We conduct the blocking assay with the four set-ups in a “dose-dependent” manner by 3-fold serial dilution of antibodies. We have repeated this experiment for 3 times and the readout are relatively stable. The “blocking” activity is stronger in the stalk region group comparing to the GS-linker or stalk-deletion group. However, partial blockage exists in the GS C-tag group which means if we replace the stalk region with smaller side chain residues, the blocking effect are weaken but not totally abolished. This observation may further demonstrate that the stalk region really matters.

The quantification of assay MFI along with appropriate statistics are also included in the figures.

We rewrote some part of the results of this section as well as the method sections. Hope this will make it easier to read.

2. The title of Fig.4 claims D250 is a key binding site, however the authors own mutagenesis shows mutation of D250 has minimal to no effect on binding. The reviewer recognizes this is a site of overlap between both mAbs, but the authors have not demonstrated this is a critical residue that mediates binding. The claim should be tempered or re-written.

Thank you for pointing this out, we have modified the title accordingly.

3. The “inhibition of the fusion loop” is a strong claim. The FFWO assay simply shows the antibodies block fusion. I don’t think the authors can really make the claim that these antibodies block exposure of the fusion loop. The claims should be tempered.

We have modified the claims to “inhibit the exposure of fusion loop.” This claim is also mentioned in some previous publications based on the structure of alphaviruses. With the B domains tethered, it is more difficult for the fusion loops in E1 to be exposed. So, antibodies stabilizing the A-B strand and E2-DB like CHK-265, CHK-263 etc. are all considered to inhibit the exposure of fusion loop.

Minor:

1. Does the CHK-263 epitope exactly overlap with C34 and C37? While C34 and C37-like antibodies have been characterized, a possible point of novelty that could be highlighted.

We appreciate your suggestion on novelty here. CHK-263 is a mouse antibody, and is not covering a fully overlapped epitope of C34 and C37. A major difference is that in C34 and C37, the heavy chain is more adjacent to the virion surface, while in CHK-263, it is the light chain to be adjacent to the virion surface. One consequence is that C34 and C37 could still crosslink various virion while CHK-263 could only form intra-virion crosslinking. We reported this phenomenon in Fig 3o and will include a short discuss from the structural aspects in this section.

2. The construct encoding the E1-E2 FL protein is still quite unclear, the citation in the method refers to the Song et al Cell 2019 paper which used p62-E1. Could the authors expand the methods to describe the exact residues and linkers used to construct this molecule.

We have updated the detailed construction in methods.

3. The flow cytometry plots would benefit from labelling of what molecule is being measured in addition to fluorochrome used to help orient the reader.

We have updated the labelling to point out the molecule being measured in the revision.

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my comments. I have one small and final suggestion in relation to a previous comment about the theoretical limits for intravirion crosslinking.

Line 287-289. 'For C37 Fab molecules around the 2f vertex, all measured distances were within the 50 Å theoretical radius for intravirion crosslinking while slightly above 50 Å for C34 (Fig. 3g-h, k-l,k).'

Can I suggest that the authors include a reference here introducing this theoretical radius for intravirion crosslinking either in the case of CHIKV or some other related virus? Or describe how they calculate the number themselves.

We appreciate the suggestions. We have modified the descriptions on intravirion crosslinking radius and updated with a reference. (ref 50)

Reviewer #4 (Remarks to the Author):

The manuscript is improved.

I appreciate the dose-titration of mAb blockade and transparency of data display - however again in Fig 5E (left two panels) the MFI between no stalk and stalk are markedly different. The right two panels showing stalk and GS-linker show more similar MFI of binding, and here the difference in blockade is reduced. Statistics are still missing.

We have updated the statistics for highest concentration of each mAb against SKT-20 which served as a negative control.