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Peer Review File

Human monoclonal antibodies that target clade 2.3.4.4b H5N1 hemagglutinin

Corresponding Author: Professor Florian Krammer

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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)
The authors have addressed the comments of the reviewers by elaborating in the text and including additional figures/tables. They have opted not to perform any additional experiments that would have been very informative and better address some of the comments.

Reviewer #2

(Remarks to the Author)
Reviewer #2 is now pleased with the authors' responses and revised version of the data and the manuscript.

Reviewer #3

(Remarks to the Author)
The authors have addressed all my previous concerns.

Reviewer #4

(Remarks to the Author)
The authors have addressed all of my comments.

We would like to thank the reviewers for their helpful comments. We have addressed their points below point by point. Line numbers refer to the redlined pdf version of the revised manuscript.

Referee expertise:
Referee #1: bNAbs, influenza, host response
Referee #2: influenza virology, host response, antibodies
Referee #3: structural biology, virology
Referee #4 (co-reviewed with Ref. 3): structural biology, virology

Referees' comments:

Referee #1 (Remarks to the Author):

Alzua et al describe the identification of monoclonal antibodies against avian influenza 2.3.4.4b. They used a humanized mice vaccination model to obtain HA-specific antibodies against H5. They isolated and described a set of 5 clonal families with prophylactic and therapeutic potential. Overall, the study addresses an important and timely topic. The manuscript is well-written, however there are some open questions as stated below.

Major comments:

- The title suggest that the antibodies have a human origin, however they are from humanized mice and stating "human monoclonal antibodies" in the title is misleading.

Response: We respectfully disagree with the reviewer. After consulting with a number of B-cell biologists we were assured that 'human monoclonal antibodies' would be their choice of words here too since 'humanized' typically refers to mouse antibodies that were converted to 'humanized' antibodies via grafting of the CDRs. The consensus was that 'humanized' would be misleading as well. Further, antibodies from transgenic animals which are being developed as clinical candidates are often characterized as fully human as opposed to humanized. We therefore elected to keep the title as is.

- In the abstract and discussion / conclusions it should be stated that these mAbs are not effective against the other H5 strain that is currently causing human infections; 2.3.2.1c.

Response: We added a statement that the antibodies would not be effective against clade 2.3.2.1c (which has caused a handful of human infections in Southeast Asia – in contrast to more than 70 human infections with clade 2.3.4.4b). See line 225-227.

- No direct comparison have been made with known H5 reactive mAbs such as very broad stem mAbs.

Response: That is correct. We focused on the mAbs we discovered. We are planning to compare these head-binding mAbs to stalk- and NA- binders in the future and we are also planning to test mAb combinations as described in the manuscript.

- The structure analysis showed binding to the hydrophobic groove, however which residues is not

mentioned in the text and if this is variable between the different mAbs. The sequence conservation analyses revealed that this hydrophobic groove is conserved with in the 2.3.4.4 strains but not other H5 strains such as 2.3.2.1c. The dependence of the mAbs for these specific residues that are different between these two lineages of H5 should be shown by neutralization and binding experiments against mutants. What is the threshold for viral escape of these mAbs, did you observe mutations in the therapeutic or prophylactic mice experiments?

Response: The discussion of residue contacts has been significantly expanded and now includes all non-van der Waals interactions that are computationally predicted. To assist with clarity, the conservation of the hydrophobic groove and other antigen residues involved in antibody engagement is now presented in Figure 5E using an alignment of 2.3.4.4b strains only, with a full sequence alignment added to the supplementary materials for ease of visualization by the reader (Supplementary Figure 9).

- Why was 1x LD50 used in the first round of mice experiments depicting weight loss and 5x LD50 when evaluating viral load and therapeutic potential. This way the weight loss and viral loads cannot be linked and would weight loss be different when using a 5x higher challenge dose?

Response: Apologies for the mistake. It was 5 x LD50 in both cases.

Minor comments:

- It is some what unclear why certain viral strains were used for specific experiments.

Response: We have amended the text to make this clearer. See line 286-288.

- The added value of Fig 1c is unclear. The data are very similar across the different assays, so why not show the data just as neutralization in a similar way as Fig 1A and 1B?

Response: The different modes of the assay did not give us large differences. However, there were differences and they would be hard to see using heat maps but can be appreciated using the bar graphs. We therefore elected to keep Figure 1C as is.

Referee #2 (Remarks to the Author):

In this study, the authors isolated a panel of monoclonal antibodies by vaccinating antibody variable region-humanized mice with adjuvanted recombinant H5HA (clade 2.3.4.4b) and N1NA proteins. They screened >1,900 clones and identified 401 potential candidates that were cross-reactive against several clade 2.3.4.4b H5HAs. The antibody clones were not necessarily reactive against H5HAs from different antigenic clades. Sequential analysis revealed that the down-selected 16 clones could be categorized into five different clonotypes, which showed some variety in HA-binding breadth and avidity. The authors also examined the protective effects of the antibodies from the five different clonotypes in a mouse challenge model and found that most of them, except the clone from 'clonotype #4', protected mice from lethal H5 virus challenge with varied protective potency, demonstrating that the binding modes of these antibodies contribute to in vivo protection. Further structural analysis revealed that similar epitopes were recognized by some of the candidates, even though the clonotypes were different. There

are several points to be addressed in the manuscript.

Specific points:
- Is there a specific reason why the intraperitoneal route was chosen for recombinant protein immunization, rather than the intramuscular or subcutaneous route? If there is a reason, it could be mentioned in the Methods section.
Response: There is no specific reason intraperitoneal injections were chosen for this project except as a part of our standard operating protocol. In our experience, we have found intraperitoneal injections anecdotally to be more optimal to intramuscular or subcutaneous in stimulating splenocytes for B cell hybridomas especially with soluble viral proteins, with or without adjuvants. This is based on our own extensive experiences over many years and has been consistent with the hybridoma technology platforms we utilize. See line 299-300.

- This reviewer is wondering how general and/or broadly applicable the findings about the features of the antibodies in this study are, given that the antibodies isolated and characterized were from a single immunized animal. This point could be discussed in the Discussion section.
Response: We completely agree with the reviewer. The point of the work was to generate mAbs that could be useful as therapeutics or prophylactics, rather than to analyze the specific immune response of these mice to clade 2.3.4.4b HA. We changed the discussion and introduction to make that clearer. See line 221-223.

- Lines 123–124: The authors could conduct an NAI assay based on soluble-form NA ligands such as MUNANA, or with NA-bearing VLPs, to see if the NAI activity of the antibodies can be attributed to steric hindrance.
Response: Good point. We conducted the NA-Star assay, and the data are included in Supplementary Figure 3. The assay methods are described in Supplementary Material. The results are discussed in the main manuscript, lines 127-129.

- Do the authors think that the “clonotype #4 antibodies” are false positives due to technical reasons? If so, this could be mentioned in the Discussion section to aid in reader understanding.
Response: We do not believe it was a false positive but rather a poor binder. We have added text to the discussion section to describe this. See line 233-236.

- Lines 224–226: The authors should test whether the clonotype 1 antibody and one of the clonotype 2, 3, or 5 antibodies work synergistically without steric hindrance. Also, do the NI activity-positive antibodies in this study not compete with anti-NA antibodies because of steric hindrance?
Response: Very good point. We are providing this data now in Supplementary Figure 4. Regarding the HA versus NA antibodies, since they bind to different proteins steric hindrance there is unlikely.
The assay methods are described in Supplementary Material.

- Lines 221 and 223: “12D10” should be “20D10”. **Corrected.**

- Lines 221: “12D20” should be “20D10”. **Corrected.**
- Line 264: “interperitoneally” should be “intraperitoneally”. **Corrected.**
- The details of the recombinant protein construction (HA or NA expression plasmids) should be included in the Methods section (i.e., what kind of tags, stabilizing motifs or amino acid substitutions were used for the immunization or ELISA antigen, respectively).

Response: Thank you, this has been included in the Materials and Methods section in the main manuscript.

- Line 524: “Binding breath” should be “binding breadth”

Response: Thank you, these changes have been made.

Referee #3 (Remarks to the Author):

In this study, Alzua et al. isolated a panel of human mAbs against influenza H5 HA by immunizing humanized mice with recombinant H5 and N1 proteins, followed by hybridoma technology. This resulted in the identification of 16 mAbs from 5 clonotypes that strongly bind H5 HA with HI activity. Most of these 16 mAbs showed strong binding to H5 HAs from different subclades of 2.3.4.4, but not to H5 HAs from other clades. Also, none showed cross-reactivity to an H1 HA. These mAbs with strong binding also inhibited viral entry and egress and exhibited NA inhibition activity, which are features of HA head antibodies (PMID: 24348996). The top neutralizing antibodies also provided protection against H5N1, both prophylactically and therapeutically, in a mouse model. In the end, the cryo-EM structures of three of the top neutralizing antibodies were solved, although their resolutions were not very high (all >3 A). All three antibodies utilized a "LxYxY" motif to interact with the hydrophobic groove. While this study is technically sound, its novelty and impact are limited, as many studies have already described the isolation and characterization of H5-neutralizing mAbs, including some that are very broadly reactive (PMIDs: 19079604, 21798894, 22238297, 22878502, 23870317, 26170284, 26635249, 27910950, 30154240, 39854479). The limited breadth of the mAbs in the present study also represents a major weakness.

Major comments:

1. Compared to 20D10 and 12G1, 1A1 has better protection prophylactically, but worse protection therapeutically. What could be the reason for this discrepancy?

Response: We believe that the half-life on the different antibodies could play a role here. We have included this speculation into the discussion section. See line 232-233.

2. It is unclear what is the advantage of the mAbs isolated in this study compared to other existing human mAbs that can target H5 HA with higher breadth, including both head antibodies (e.g. FLD194 and 65C6) and stem antibodies (e.g. CR9114, CR6261, FI6v3, 39.29, and 3E1).

Response: It is currently unclear if FLD194 or 65C6 bind to clade 2.3.4.4b HA. Stalk-reactive antibodies have been shown to bind and could be used in combination with head-reactive antibodies shown here.

3. The authors proposed that the mAbs reported in this study could serve as treatments during a H5N1 epidemic or pandemic. However, given the limited breadth of these mAbs, escape mutations will likely be a problem. The authors should test how easy or difficult it is for the virus to escape their mAbs.
Response: We agree with the reviewer's statement and have contemplated these experiments, which are easy to do. However, they would be GoF experiments (especially under the new NIH notice), even if performed with VSV-H5, and due to the current political climate and public perception, we decided not to move ahead with them.

Minor comments:
1. Line 63: missing full stop after references 8 and 9?
Response: Thank you. Corrected.

2. Line 94: "These mAbs were expressed …" Does that mean recombinant expression, which is different from the hybridoma expression mentioned earlier (line 71)?
Response: Clarified.

3. Lines 181-182: "the 6G1 cryoEM sample was co-complexed with mAb 1A1 (clonotype 1)" it is unclear what this means. Did the authors first form the 6G1-HA complex and then add 1A1 to it? This doesn't make sense, though, since 1A1 and 6G1 would compete for binding.
Lines 182-197: Based on preliminary negative staining data it appeared that the epitopes targeted by 6G1 and 1A1 would be sufficiently distinct to allow for simultaneous binding when complexing H5 with Fabs of 6G1, 1A1, and CR9114 (as a fiducial marker) followed by computational differentiation via focused classification and refinement on the densities for 6G1 and 1A1. While we were able to observe binding of both 6G1 and 1A1 from one dataset, it is true that we were only able to resolve 6G1 to sufficient resolution for model building and that a solo complex of 1A1 may have yielded better results.

4. Figure S4: should "20D1" be "20D10"?
Response: Corrected.

5. Figure 4F: It is unclear how the sequence conservation was calculated. The scale of the color bar is also not labelled.
6. Lines 193-194: "we found that the hydrophobic groove is well conserved". Based on Figure 4F, the pocket that Y100E binds to seems quite highly variable (dark color).
7. Lines 195-196: "amongst the identified interacting residues, I166 is particularly variable." The authors need to show the data for this.
Figure 4F, lines 193-194, and lines 195-196: The surface colored by conservation has been moved to Figure 5E. While the figure still presents a scale bar with "more conserved" and "less conserved" for ease of interpretation, the AL2CO scores for positional conservation are listed in the figure legend. The sequence alignment used to generate Figure 5E now only includes strains from 2.3.4.4b and is labeled