

Structurally Convergent Antibodies Derived from Different Vaccine Strategies Target the Influenza Virus HA Anchor Epitope with a Subset of VH3 and VK3 Genes

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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the manuscript "Structurally Convergent Antibodies Derived from Different Vaccine Strategies Target the Influenza Virus HA Anchor Epitope with a Subset of VH3 and VK3 Genes" Lin et al. determine high resolution structures of antibodies bound to a conserved anchor epitope (membrane-proximal region) in the influenza HA protein. The antibodies were elicited in different individuals through vaccination using different regimen. Although the anchor epitope has been described (references 35, 39 and 40) and several features of antibody interactions of this epitope were recognized before (such as the importance of the LCR3 NWPP motif), this study provides a set of additional antibody-bound structures and also delves into the mechanistic aspects of antibody recognition of this epitope through complementary approaches including structure guided mutagenesis coupled with binding studies.

My one major issue was that the organization of the manuscript made it rather tedious to read. I would highly recommend a reorganization to combine similar views from the different structures and show them side by side. For example, panel d from Figures 3-6 would be useful to see side-by-side. Similarly for other panels.

Extended data figure 10 should be expanded to include a representative micrograph, representative 2D class averages, and representative views of maps with underlying fitted model.

Extended Data Figure 7: The fits to the BLI plots in the top panels are suboptimal. These measurements and analyses should be improved.

BLI raw data and fitted curves should be shown throughout (for example, for the data shown in Extended Data Figure 4.)

Line 176-177: "Y92, the only SHM in LCDR3, interacts with β -strands on HA2 and hydrogen bonds with G16 in the fusion peptide (Fig. 3d)." The β -strands would be better visualized in cartoon representation than as a surface.

Line 238-240: "These findings are consistent with the alanine scanning (Extended Data Fig. 4) and provide insights into the importance of different SHM residues in HCDR2, and some flexibility in anchor epitope recognition." This should be rephrased, as flexibility may be misconstrued to mean positional flexibility, whereas what the authors likely mean here is that the interaction tolerates some variability in the antibody sequence.

Line 378-379: "However, 222-1C06VH with 346-54VK showed weak binding to A/Michigan/45/2015 H1 HA (Extended Data Fig. 7)." Extended Data Fig 7 legend says that the binding data shown are with A/California/04/2009 H1N1 HA.

Minor comment:

Line 93: "Furthermore, these antibodies have a wide spectrum of binding and neutralizing to the H1N1 subtype." should probably be "Furthermore, these antibodies have a wide spectrum of binding and neutralizing activities to the H1N1 subtype."

Reviewer #2

(Remarks to the Author)

This paper addresses influenza hemagglutinin (HA) binding antibodies which bind near to the membrane anchor. This paper examines antibodies from a number of sources which all bind to an epitope on HA, which is very similar to the previously published FISW84 antibody. This range of antibodies are interesting, as they highlight that antibodies that bind to this epitope are likely commonly occurring either from vaccination or infection. These new antibodies are also broadly neutralising of a wide range of H1 HAs, as seen previously with FISW84. This epitope could be interesting for tuning vaccines towards, as it is on an area of HA with low sequence variability. The results in this paper are interesting, but largely descriptive and consequently don't do a great deal to advance our mechanistic understanding of HA neutralisation.

The paper consists of structures, either X-ray or cryoEM of these antibodies bound to HA. These experiments are competently done and support the structural descriptions contained within the paper and are well described to allow reproducibility.

This class of antibody is interesting and the physical constraints of them binding to HA in a membrane is a little unclear. The paper could do with discussing this in greater detail, as the previous paper with FISW84 found better neutralisation of the fAb fragment when compared to the IgG.

Reviewer #3

(Remarks to the Author)

Lin et. al. report the structural and functional characteristics of antibodies targeting the anchor epitope of influenza virus hemagglutinin (HA). The study focuses on four mABs derived from different vaccination strategies and examines their binding modes, genetic origins, and neutralization ability against H1N1 influenza strains. The study is well-designed and provides valuable insight into influenza vaccine design and the potential role of anchor epitope-targeting antibodies.

Some areas that should be addressed to improve the work include:

- 1) A great deal of the focus of the research is on the conservation of germline gene usage in recognizing the anchor epitope. What is not clear from the discussion is what exactly the significance is of this finding. As written the reader is given the impression that this is surprising, but it is unclear from the literature analysis if convergent germline gene usage in binding a common epitope is surprising, unexpected or rather is common when many antibodies against a single epitope are examined. I am certain there are many examples of this in the literature/structural database that could be drawn on.
- 2) Additional details regarding the demographics and genetic background of the donors from which these antibodies were derived would be useful information. If all of the donors come from the same "types" of people (for eg. all European descent) perhaps it's less surprising that common that similarly germline genes were used. The use of mABs isolated from a highly diverse population with wide variety of genetic backgrounds would be much more informative regarding germline gene usage. If the backgrounds of the donor were homogenous, or are unknown this should be stated and the caution taken with conclusions about germline gene restriction for recognizing this epitope
- 3) The selection criteria for antibodies used in the study should be explained in more detail. Among the many antibodies available to the authors for study, why were these 4 selected?
- 4) Direct comparison of the Abs in this study with established bnAbs (e.g., CR9114) in functional/neutralization assays might be useful for providing some additional context for the efficacy of the anchor antibodies
- 5) Perhaps I missed it, but what is the purpose and significance of the thermal stability assay?
- 6) The methods should include a description of how exactly the germline gene analysis was carried out
- 7) In the text and in extended data Fig 1, the authors report mutations in the sequences and state that these are the result of SHM. Related to item 6 above, I presume this was concluded based on comparing the sequences to ref germlines in IMGT or other databases. How do the authors know that the sequence differences are the result of SHM and not either sequencing errors or natural genetic diversity? I believe caution must be taken before concluding that sequence differences are the result of SHM.

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Reviewer #1 (Remarks to the Author):

In the manuscript “Structurally Convergent Antibodies Derived from Different 1 Vaccine Strategies Target the Influenza Virus HA Anchor Epitope with a Subset of VH3 and VK3 Genes” Lin et al. determine high resolution structures of antibodies bound to a conserved anchor epitope (membrane-proximal region) in the influenza HA protein. The antibodies were elicited in different individuals through vaccination using different regimen. Although the anchor epitope has been described (references 35, 39 and 40) and several features of antibody interactions of this epitope were recognized before (such as the importance of the LCR3 NWPP motif), this study provides a set of additional antibody-bound structures and also delves into the mechanistic aspects of antibody recognition of this epitope through complementary approaches including structure guided mutagenesis coupled with binding studies.

Response: We thank the reviewer for the positive comments and for providing very helpful and thoughtful suggestions on the manuscript.

My one major issue was that the organization of the manuscript made it rather tedious to read. I would highly recommend a reorganization to combine similar views from the different structures and show them side by side. For example, panel d from Figures 3-6 would be useful to see side-by-side. Similarly for other panels.

Response: We agree and thank the reviewer for the suggestion.

We have completely reorganized Figures 3-6 that are now Figures 2-4. We combined the four overall structures with the antibody epitopes highlighted with the details of the interaction of CDR loops for each Fab with their cognate epitopes to Figure 2 and reorganized the germline gene comparisons of the heavy chain and light chain to Figures 3 and Figure 4.

Extended data figure 10 should be expanded to include a representative micrograph, representative 2D class averages, and representative views of maps with underlying fitted model.

Response: We thank the reviewer for this comment and have added a representative micrograph, representative 2D classes, 3D classes, and the model fit in the map in **Extended Data Figure 10**.

Extended Data Figure 7: The fits to the BLI plots in the top panels are suboptimal. These measurements and analyses should be improved.

Response: We thank the reviewer for this comment.

We have remeasured the binding of 047-09_4F04 $V_H + 346-54 V_K$, 241_2F04 $V_H + 346-54 V_K$, and 346-54 $V_H + 047-09_4F04 V_K$ with CA04 HA and have updated **Extended Data Figure 7** accordingly.

BLI raw data and fitted curves should be shown throughout (for example, for the data shown in Extended Data Figure 4.)

Response: We thank the reviewer for this suggestion.

We have added the Sensorgrams for binding of 047-09_4F04 WT, 241_2F04 WT, 346-54 WT, SFV009_3G01 and 222-1C06 and their mutants to CA04 HA in **Extended Data Figures 11-14**.

We also added the Sensorgrams for binding of 047-09_4F04 WT, 241_2F04 WT, 346-54 WT, SFV009_3G01 and 222-1C06 to CA04 HA with conserved H3N2 HA residue and alanine substitutions in anchor epitopes in **Extended Data Figures 15 and 16**.

Line 176-177: “Y92, the only SHM in LCDR3, interacts with β -strands on HA2 and hydrogen bonds with G16 in the fusion peptide (Fig. 3d).” The β -strands would be better visualized in cartoon representation than as a surface.

Response: We thank the reviewer for pointing out this issue.

We apologize - this was a typo. Y92 does not interact with the β -strands on HA2 but instead interacts with G16 in the fusion peptide. We have corrected in the revised sentence (lines 187-188).

“Y92, the only SHM in LCDR3, hydrogen bonds with G16 in the fusion peptide (Fig. 4b).”

Line 238-240: “These findings are consistent with the alanine scanning (Extended Data Fig. 4) and provide insights into the importance of different SHM residues in HCDR2, and some flexibility in anchor epitope recognition.” This should be rephrased, as flexibility may be misconstrued to mean positional flexibility, whereas what the authors likely mean here is that the interaction tolerates some variability in the antibody sequence.

Response: We thank the reviewer for the suggestion, and we agree and have changed flexibility to variation (lines 262-265).

“These findings are consistent with alanine scanning (Extended Data Fig. 4) and provide insights into the importance of different SHM residues in HCDR2 that show some variation in anchor epitope recognition.”

Lines 378-379: “However, 222-1C06VH with 346-54VK showed weak binding to A/Michigan/45/2015 H1 HA (Extended Data Fig. 7).” Extended Data Fig 7 legend says that the binding data shown are with A/California/04/2009 H1N1 HA.

Response: We thank the reviewer for pointing out this mistake. We have corrected the Figure legend and added A/Michigan/45/2015 H1 HA in **Extended Data Fig. 7**.

“Binding kinetics of 047-09_4F04 V_H , 241_2F04 V_H , 346-54 V_H , and SFV009_3G01 V_H anchor antibodies expressed with different V_K genes to A/California/04/2009 H1N1 HA were measured by (BLI). Binding kinetics of 222-1C06 V_H anchor antibody expressed with different V_K genes to A/Michigan/45/2015 H1 HA. Black lines represent the response, and gray dashes represent the 1:1 binding model. Binding affinities were measured at 2-fold serial dilution of Fab starting from 500 nM. $K_D > 1000$ nM represent binding detected only in the highest concentration. See Fig. 1 for WT binding.”

Minor comment:

Line 93: “Furthermore, these antibodies have a wide spectrum of binding and neutralizing to the H1N1 subtype.” should probably be “Furthermore, these antibodies have a wide spectrum of binding and neutralizing activities to the H1N1 subtype.”

Response: We thank the reviewer for this suggestion.

We have revised this sentence in lines 93-94.

“Furthermore, these antibodies have a wide spectrum of binding and neutralizing activities to the H1N1 subtype.”

Reviewer #2 (Remarks to the Author):

This paper addresses influenza hemagglutinin (HA) binding antibodies which bind near to the membrane anchor. This paper examines antibodies from a number of sources which all bind to an epitope on HA, which is very similar to the previously published FISW84 antibody. This range of antibodies are interesting, as they highlight that antibodies that bind to this epitope are likely commonly occurring either from vaccination or infection. These new antibodies are also broadly neutralising of a wide range of H1 HAs, as seen previously with FISW84. This epitope could be interesting for tuning vaccines towards, as it is on an area of HA with low sequence variability. The results in this paper are interesting, but largely descriptive and consequently don't do a great deal to advance our mechanistic understanding of HA neutralisation.

The paper consists of structures, either X-ray or cryoEM of these antibodies bound to HA. These experiments are competently done and support the structural descriptions contained within the paper and are well described to allow reproducibility.

This class of antibody is interesting and the physical constraints of them binding to HA in a membrane is a little unclear. The paper could do with discussing this in greater detail, as the previous paper with FISW84 found better neutralisation of the fAb fragment when compared to the IgG.

Response: We thank the reviewer for the positive comments on the manuscript. We added more details regarding structurally convergent features from different germplines (lines 560-568).

“Thus, the structurally convergent features derived from different V_H (V_{H3-23} , V_{H3-30} , $V_{H3-30-3}$, and V_{H3-48}) and V_K (V_{K3-11} and V_{K3-15}) germplines appear to be a critical factor in antibody targeting of this conserved epitope, as also demonstrated by antibodies to SARS-CoV-2 (YYDRxG in HCDR3)⁵³ and HIV (CD4 binding site)⁵⁴. Notably, 346-54, isolated from a chimeric HA vaccine trial¹⁶, has more variation in HCDR2 but still retains a similar binding mode, and displays neutralizing breadth and potency that rivals FI6v3 in H1N1 viruses²⁵, suggesting some amino acid tolerance in this convergent antibody structure.”

Reviewer #3 (Remarks to the Author):

Lin et. al. report the structural and functional characteristics of antibodies targeting the anchor epitope of influenza virus hemagglutinin (HA). The study focuses on four mABs derived from different vaccination strategies and examines their binding modes, genetic origins, and neutralization ability against H1N1 influenza strains. The study is well-designed and provides valuable insight into influenza vaccine design and the potential role of anchor epitope-targeting antibodies.

Some areas that should be addressed to improve the work include:

1) A great deal of the focus of the research is on the conservation of germline gene usage in recognizing the anchor epitope. What is not clear from the discussion is what exactly the significance is of this finding. As written the reader is given the impression that this is surprising, but it is unclear from the literature analysis if convergent germline gene usage in

binding a common epitope is surprising, unexpected or rather is common when many antibodies against a single epitope are examined. I am certain there are many examples of this in the literature/structural database that could be drawn on.

Response: We thank reviewer for the great suggestions and comments.

We added further discussion in lines 560-564.

“Thus, the structurally convergent features derived from different V_H (V_{H3-23} , V_{H3-30} , $V_{H3-30-3}$, and V_{H3-48}) and V_K (V_{K3-11} and V_{K3-15}) germlines appear to be a critical factor in antibody targeting of this conserved epitope, as also demonstrated by antibodies to SARS-CoV-2 (YYDRxG in HCDR3)⁵³ and HIV (CD4 binding site)⁵⁴. ”

2) Additional details regarding the demographics and genetic background of the donors from which these antibodies were derived would be useful information. If all of the donors come from the same “types” of people (for eg. all European descent) perhaps its less surprising that common that similarly germline genes were used. The use of mAbs isolated from a highly diverse population with wide variety of genetic backgrounds would be much more informative regarding germline gene usage. If the backgrounds of the donor were homogenous, or are unknown this should be stated and the caution taken with conclusions about germline gene restriction for recognizing this epitope.

Response: We thank the reviewer for this suggestion. However, only information such as age and gender were described in the published paper on isolation of these antibodies (ref. 36) by our collaborators. These studies. However. were conducted in the US. We have added a brief information in material and methods (lines 634-637).

“Anchor antibodies listed in Extended Data Fig. 1 and selected in this study were isolated from participants immunized with 2009 monovalent inactivated pandemic influenza vaccine (MIV), 2004-2015 quadrivalent inactivated influenza vaccine (QIV) or chimeric cH8/1 and cH5/1 influenza A group 1 cHA vaccine in prior studies^{16,17,36}. ”

3) The selection criteria for antibodies used in the study should be explained in more detail.

Among the many antibodies available to the authors for study, why were these 4 selected?

Response: We thank the reviewer for this suggestion and have incorporated a corresponding description in the results section (lines 105-107).

“To identify the epitopes and binding mode of anchor antibodies, we selected four antibodies encoded by distinct germline genes and one with the same germline as the previously published 222-1C06 anchor antibody for comparison.”

4) Direct comparison of them Abs in this study with established bnAbs (e.g., CR9114) in functional/neutralization assays might be useful for would providing some additional context for the efficacy of the anchor antibodies

Response: We thank the reviewer for this suggestion.

However, antibody FI6V3 used in our neutralization assays is also a well-known and established bnAb to the central stem epitope with extensive breadth. Both FI6V3 and CR9114 are capable of neutralizing group 1 and group 2 influenza A viruses.

5) Perhaps I missed it, but what is the purpose and significance of the thermal stability assay?

Response: We used thermal stability to test the important conformation of cis-proline in NWPP motif in LCDR3. This motif binds to the same position on HA via a W-shape conformation in all anchor antibodies.

We explain more clearly in lines 339-353.

“To decipher why A95a could cause such a dramatic effect on binding to HA, we determined an apo crystal structure with A95a in 241_2F04 (241_2F04-A95a). After superimposition of 241_2F04-A95a onto 241_2F04-HA complex, we observed a substantial movement in the NWPP region, with W94 flipping ~ 90° as the cis conformation at A95a was now abolished (Fig. 5b). The flipped W94 would then be close to HCDR3 likely perturbing the conformation of HCDR3, which resulted in a slight reduction in protein stability in a thermal shift assay (Fig. 5b and 5e).”

6) The methods should include a description of how exactly the germline gene analysis was carried out

Response: We thank the reviewer for this suggestion. We have added the method for germline gene analysis in material and methods (lines 743-747).

“Immunoglobulin heavy and light chain sequences analysis

Germline gene usage, complementarity-determining regions (CDRs) analysis, VDJ genes rearrangement, and sequence identity to its germline for each anchor antibody were analyzed in IgBLAST⁷² and international ImMunoGeneTics information system (IMGT)⁷³. Kabat numbering analysis of each Fab was carried out by Abysis⁷⁴. ”

7) In the text and in extended data Fig 1, the authors report mutations in the sequences and state that these are the result of SHM. Related to item 6 above, I presume this was concluded based on comparing the sequences to ref germlines in IMGT or other databases. How do the authors know that the sequence differences are the result of SHM and not either sequencing errors or natural genetic diversity? I believe caution must be taken before concluding that sequences differences are the result of SHM.

Response: We did indeed compare the antibody sequences to reference germlines in IMGT. We were able to assign not only the germline but also the specific allele. Hence, for the germline sequences, we can be fairly confident about SHM from the germline. We also understand the concern and do not wish over-interpret the sequences difference. However, if we had not been able to assign germline and allele, we would have certainly been more cautious in SHM designation. We and others have routinely used this approach in many other published papers.