nature portfolio

Peer Review File

The asymmetric opening of HIV-1 Env by a potent CD4 mimetic enables anti-coreceptor binding site antibodies to mediate ADCC

Corresponding Author: Dr Andrés Finzi

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)
et al study an improved CD4 mimetic that enhances HIV-specific ADCC using otherwise unhelpful antibodies. The work is well done using a number of viral strains and structural data.

Comments

The impact of the work would be greatly improved if the authors could show a functional difference in an animal model of HIV infection. Otherwise it is unclear whether this in vitro improvement is meaningful.

A schematic diagram of how this is working would help the general reader.

Figure 1a and 1b: It's unclear whether the anti-cluster A antibody data here represent either A32 or N5i5, or the average of the two mAbs. Were they both used in combination with 17b or just one of them?

Figures 2 and 3 should indicate which mAbs are being used (is it just 17b alone and 17b+A32?).

For Figures 2 and 3, it is interesting that for CJF-III-288 treated cells, there is much higher antibody binding when A32 and 17b are used in combination compared to the 17b alone, but this does not translate to higher ADCC (compare Fig 2h to 2f or 3h to 3f). Is A32's Fc portion somewhat blocked when in combination with 17b, leading to no improvement in ADCC despite the higher binding?

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
Richard et al. present a manuscript describing sensitization of HIV-1 infected cells to ADCC by a CD4-mimetic compound, CFJ-III-288, through anti-co-receptor binding site antibodies. Comparing the effect of this compound to a similar compound,

BNM-III-170, the authors show that, while both compounds open the Envelope to anti-co-receptor binding site antibodies, CFJ-III-228 is shifts the conformational distribution to a greater degree. This is correlated with greater ADCC activity for co-receptor binding antibodies compared to BNM-III-170 triggered Envs. The authors present a cryo-EM structure of the a BG505 Env SOSIP ectodomain bound to both CFJ-III-228 and the anti-co-receptor binding site antibody Fab 17b, comparing the conformation to published liganded and unliganded BG505 structures in various conformations, including a structure of BG505 bound to BNM-III-170 and 17b. Additionally, cryo-ET of inactivated viruses bound to 17b Fab and either CFJ-III-228 and BNM-III-170 are presented to support findings from the single particle cryo-EM work. While the results are interesting, the interpretation of the structures and the presentation of the smFRET have important issues that must be addressed. The authors show correlation between ADCC activity and the conformations induced. However, the data suggests additional factors are involved. The structure fits and interpretations are also unclear and a rationale for differences in Fab binding orientation giving rise to the observed ADCC differences is tenuous. A plausible mechanism for how a shift in the angle of the epitope changes ADCC activity is not presented.

My specific comments are as follows:

• The authors state on lines 328-330 that the small molecules induce conformations that alter the angle of approach for 17b. However, the structures include the 17b Fab and therefore cannot determine whether the shifted positions are related to a coupled 17b-compoud effect or are do only to the compound.
• The authors perform alignments and measure the distances between the gp41 helices and the alpha-0 helices in the gp120s to report changes in the position of the gp120 domains relative to gp41. First, this is not adequately described in the results or in the methods section. The specific residue or residues for this need to be described, including the atom(s) select for the different analyses (this can be placed in the main text or the methods section). The authors must also modify the language used to describe the differences observed. On line 307, the authors state that the alpha-0 “rotates” by different distances for each protomer. This needs to be modified to indicate that the alpha-0 is displaced by those distance or an angular term needs to be used to describe the shift. I suggest measuring an angular displacement based on a hinge point; distance measures are nevertheless common for examining changes in gp120 disposition and are acceptable.
• The authors present several measurements based on the structures to argue that the 17b Fab binding orientation has shifted in the CFJ-III-228 liganded structure compared to the BNM-III-170 bound structure. It is not entirely clear whether the authors mean that the epitope is presented in a different orientation or if they mean that the 17b is bound in a different orientation. The former follows from the protomer position shifts the authors have established based on alignments and distance measures (if the epitope changes position the paratope changes with it if the bound state remains the same). The authors measure the 17b binding angle of approach based on two positions, the 17b centroid and gp41. As above, a distance measure cannot be described as an angular term. Second, if the authors mean to indicate that the Fab binding orientation has changed, by measuring 17b orientation between the immobile gp41 and mobile g120, the measures will differ regardless of whether 17b positioning has shifted since it has already been established that gp120 has shifted. These measures must use gp120 and 17b points of reference to determine whether the 17b binding angle has changed. If this is not the meaning, the language requires clarification. The map fits for 17b cannot be assessed based on the figures provided but issues in the cartoon representations in extended data figure 5a suggest that the fits are poor for at least the cyan colored structure. This presumably corresponds to the lowest resolution Fab visible in panel c of extended data figure 4. At the contour presented in this figure, each antibody appears to be somewhat poorly resolved. A figure showing the fits of the entire Fab and at the paratope are needed. These are especially critical for evaluating the claim that the 17b binding orientation has shifted. Finally, considering the poor Fab resolution as the distance from the paratope increases, the authors should consider measuring using atoms that are well resolved in the Fab and the antigen to mitigate issues related to uncertain fits. If the Fab orientation has indeed shifted, this should be evident in changes to the interaction at the binding interface.
• The claims regarding 17b orientation shifts in the cryo-ET are not convincing at the resolution of these experiments unless, again, the meaning is that 17b binding follows gp120 rotations. The fit uncertainty for the models cannot resolve whether 17b has shifted position on gp120 or if the 17b position differs due to gp120 rotations about the gp41 base. The map features between the two different liganded structures are sufficient to establish the the overall rotation of a protomer-Fab complex differs, but this finer grained analysis is not supported. Together, the results are more supportive of gp120 shifting position and therefore presenting the epitope in a differing position. An unchanged 17b binding orientation results in the 17b density following the gp120 angular shift. If the authors believe this is incorrect, more careful measurement and high confidence fits in the regions used in the measurements need to be presented.
• The scale of the numbers identify the smFRET states in figure 5 a and b should be clarified. Is the scaling identical to the relative differences in state occupancy? Preferably, the number sizes would not differ so that the reader can assess the relative differences based on the data. Numerical differences provided elsewhere can be used to examine this more carefully. Figure 5e warrants further discussion in the results section. While there is a general trend toward greater ADCC activity with changing state occupancy, a clear asymptote is visible in the data over which a wide range of ADCC activities is observed despite similar conformational distributions. This suggests an additional factor that is not measured in the smFRET affects the ADCC activity.

Reviewer #4

(Remarks to the Author)
The manuscript submitted by Richard et al. is a detailed analysis on the working action of novel compounds for antiviral

therapy against HIV. The focus is on antibody-dependent cellular cytotoxicity (ADCC) which represents one of the primary effector mechanisms used by the immune system to clear infected cells. HIV-1 envelope glycoprotein (Env) trimer represents the only viral protein exposed on the surface of HIV-1-infected cells and therefore serves as the primary target for ADCC-mediating antibodies. The conformational landscape of Env is described as dynamic, where multiple states from “closed” to “fully open” are available and their population plays a key role for the action of antiviral compounds, e.g. the closed conformation is resistant to non-neutralizing antibodies. The paper shows that a newly developed compound CJF-III-288, in combination with anti-CoRBS Abs, potently stabilizes an asymmetric open conformation of Env using a combination of biochemical and biophysical experiments.

Since I am not an expert in HIV cell biology nor the structural biology of the involved protein complexes, my review focusses on the assessment on the quality of the smFRET data and their embedment into the paper. I found the article very hard to read (for the non HIV-expert) and to orient myself. In general, the involved groups have already published smFRET papers of HIV proteins which are (too) briefly summarized in the introduction. Thus, for me a comprehensive figure in the introduction with a summary on the key aspects of Env conformations would help tremendously to better understand the findings the paper, how the assay was set up etc.

The paper might have the potential for Nature Communications, yet it requires revisions and an additional expert assessment as to whether the research questions themselves have the relevance to be suitable for the journal. My comments regarding the FRET data are:

- The assignment of the states 1-4 is for me (without further information) arbitrary since it shows a continuum of all available FRET efficiency values; see Figure 5a/b

- Please determine the photon-limited width of a static inter-dye distribution to allow a clear relation of the data and the fitting model; e.g., currently the distribution of state 1 (and others) could also be related to >1 states; an unbiased approach for selecting the total number of states should be presented

- Provide expected distances based on accessible volume calculations for inter dye distances in the proposed states and compare to experimental results

- What are (were in previous publications) the effects of surface immobilization

- Do immobilization and dye attachement cause changes in the function of Env, how can this be characterized and excluded?

- Are the results obtained by smFRET independent of the used dye pair? Please provide controls.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am satisfied with the response.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
The author's updates are sufficient. Lines 353-366 are, however, verbose. Stating measures of the binding interface indicate no change in 17b contact is sufficient to support the claim that the angle of approach differs due to gp120 interface presentation.

Reviewer #4

(Remarks to the Author)
On my part of the questions no significant improvement was done during the revisions and the questions were also not answered in a satisfying way. The paper should in my opionion not be published in its current form.

- identification of states: it is unclear and without the use of a second pair of fluorophores (which could be of the same spectral characterisitics, e.g., rhodamine fluorophores) it is not clear to me if any of the smFRET data make sense

- functional controls are needed: the authors state in their resopnse: "We are therefore confident that immobilization and fluorophore attachment are not significantly impacting Env structure and function." Please provide concrete numbers (e.g., affinities +/- dye) and other concrete evidence.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
I am satisfied with the response of the authors and thank for your time to summarize their previous studies.

Please find below our point-by-point response to the reviewers' comments.

Reviewer #1 (Remarks to the Author):

Richard et al study an improved CD4 mimetic that enhances HIV-specific ADCC using otherwise unhelpful antibodies. The work is well done using a number of viral strains and structural data.

Response: We thank the reviewer for her/his positive assessment of our manuscript.

Comments

-The impact of the work would be greatly improved if the authors could show a functional difference in an animal model of HIV infection. Otherwise it is unclear whether this in vitro improvement is meaningful.

Response: As suggested by the reviewer, we now show how findings translate into an animal model. We conducted an *in vivo* study in humanized NSG-Tg(IL15) mice that express human IL15 and therefore support antibody-effector function (Fig.5). Our studies show that the CJF-III-288+17b-treatment of ART-suppressed HIV-1_{JRCSF}-infected mice at the time of analytical treatment interruption (ATI) results in delaying viral rebound in comparison to mice that were treated with BNM-III-170+17b or mock-treated. These findings support our *in vitro* studies that CJF-III-288 enables anti-CoRBS antibody-mediated ADCC of HIV-1-infected cells.

A schematic diagram of how this is working would help the general reader.

Response: we agree with the reviewer that a diagram would help the reader. We now present in Fig.10 a model summarizing our findings.

Figure 1a and 1b: It's unclear whether the anti-cluster A antibody data here represent either A32 or N5i5, or the average of the two mAbs. Were they both used in combination with 17b or just one of them?

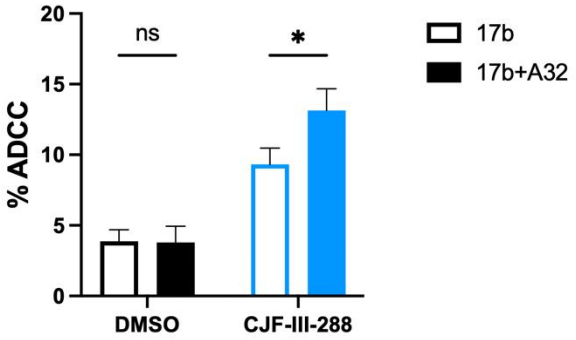
Response. We thank the reviewer for raising this. Just one of the anti-cluster A Abs, either A32 or N5i5, was used in combination with 17b. This was clarified in the corresponding figure legends.

Figures 2 and 3 should indicate which mAbs are being used (is it just 17b alone and 17b+A32?).

Response: In Figure 2 and 3. The anti-CoRBS Abs 17b was used alone or in combination with A32. This is was clarified in both figure legends.

For Figures 2 and 3, it is interesting that for CJF-III-288 treated cells, there is much higher antibody binding when A32 and 17b are used in combination compared to the 17b alone, but this does not translate to higher ADCC (compare Fig 2h to 2f or 3h to 3f). Is A32's Fc portion somewhat blocked when in combination with 17b, leading to no improvement in ADCC despite the higher binding?

Response: We thank the reviewer for this insightful question. We agree that the difference in ADCC between the condition with 17b alone and 17b in combination with A32 is much reduced in the presence of CJF-III-288 compared to BNM-III-170. This reduced contribution of A32 to the ADCC response is likely due to the ability of CJF-III-288 to enhance the ADCC activity of 17b rather than a diminished ADCC function of A32 itself. As shown in Rebuttal Figure 1, the addition of A32 to 17b, in the presence of 50 μM CJF-III-288, significantly enhanced ADCC responses against HIV-1_{CH058}-infected cells. Furthermore, in a recent study (PMID: 39248460), we reported that when A32 and 17b were combined with the anti-gp41 antibody 246D in the presence of CJF-III-288, all three non-neutralizing antibodies contributed to the elimination of HIV-1-infected cells via ADCC. Notably, we showed that introducing a leucine-to-alanine substitution (LALA mutation) at positions 234 and 235 in the Fc fragment of A32's heavy chains - known to reduce Fcγ receptor engagement - significantly reduced the ADCC activity of this nnAb cocktail. This finding rules out the possibility that A32's ADCC activity is inherently reduced in the presence of 17b and CJF-III-288.



Rebuttal Figure 1. ADCC responses detected against HIV-$1_{CH058TF}$-infected primary CD4+ T cells with 17b alone or in combination with A32, in the presence or not of CJF-III-288. Statistical significance was tested using Two-way ANOVA test with a Holm-Sidak post-test

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We are happy to contribute to the training of Early Career Researchers in peer review.

Reviewer #3 (Remarks to the Author):

Richard et al. present a manuscript describing sensitization of HIV-1 infected cells to ADCC by a CD4-mimetic compound, CFJ-III-288, through anti-co-receptor binding site antibodies. Comparing the effect of this compound to a similar compound, BNM-III-170, the authors show that, while both compounds open the Envelope to anti-co-receptor binding site antibodies, CFJ-III-228 is shifts the conformational distribution to a greater degree. This is correlated with greater ADCC activity for co-receptor binding antibodies compared to BNM-III-170 triggered Envs. The authors present a cryo-EM structure of the a BG505 Env SOSIP ectodomain bound to both CFJ-III-228 and the anti-co-receptor binding site antibody Fab 17b, comparing the conformation to published liganded and unliganded BG505 structures in various conformations, including a structure of BG505 bound to BNM-III-170 and 17b. Additionally, cryo-ET of inactivated viruses bound to 17b Fab and either CFJ-III-228 and BNM-III-170 are presented to support findings from the single particle cryo-EM work. While the results are interesting, the interpretation of the structures and the presentation of the smFRET have important issues that must be addressed. The authors show correlation between ADCC activity and the conformations induced. However, the data suggests additional factors are involved. The structure fits and interpretations are also unclear and a rationale for differences in Fab binding orientation giving rise to the observed ADCC differences is tenuous. A plausible mechanism for how a shift in the angle of the epitope changes ADCC activity is not presented.

Response: We thank the reviewer for the possibility to clarify these points.

My specific comments are as follows:

• The authors state on lines 328-330 that the small molecules induce conformations that alter the angle of approach for 17b. However, the structures include the 17b Fab and therefore cannot determine whether the shifted positions are related to a coupled 17b-compoud effect or are do only to the compound.

Response: We believe that the overall open trimer conformation observed in our studies is triggered and stabilized synergistically by both, the compound and 17b, as demonstrated by smFRET data (Figure 6a). However, our structural analyses include a comparison of two complexes of the small compound-BG505 SOSIP.664-17b Fab, which differ only in the compound used (CJF-III-170 in our study versus BNM-III-170 as reported in Jette et al., Nat Com 2021 (PMID: 33782388). Therefore, we conclude that the differences in trimer opening are most likely attributable to the specific small compound itself. Of note the structural analyses include complexes which structures were determined at similar resolution with the only difference in

the compound used. To clarify this point, we have revised the description as follows (see lines 388-395 of the "track-change" version of the manuscript):
"This analysis further demonstrates that CJF-III-288, in combination with 17b, stabilizes the Env trimer in a more asymmetric and open conformation compared to BNM-III-170 coupled with 17b. The observed changes in the overall trimer assembly are driven by alterations in the relative orientations of the gp120 protomers to gp41 within the BG505 SOSIP.664 trimer, with the most significant changes occurring in one protomer. Since the orientation of individual gp120 protomers within the trimer determines the angle of approach of 17b to the Env trimer, these angles vary for each protomer."

• The authors perform alignments and measure the distances between the gp41 helices and the alpha-0 helices in the gp120s to report changes in the position of the gp120 domains relative to gp41. First, this is not adequately described in the results or in the methods section. The specific residue or residues for this need to be described, including the atom(s) select for the different analyses (this can be placed in the main text or the methods section). The authors must also modify the language used to describe the differences observed. On line 307, the authors state that the alpha-0 "rotates" by different distances for each protomer. This needs to be modified to indicate that the alpha-0 is displaced by those distance or an angular term needs to be used to describe the shift. I suggest measuring an angular displacement based on a hinge point; distance measures are nevertheless common for examining changes in gp120 disposition and are acceptable.

Response: We thank the reviewer for these two suggestions. We modified Fig 8 to include angles rather than distances in describing differences in *α* 0 displacement relatively to central gp41 helices and have changed the text (lines 339-348 of the "track-change" version of the manuscript) to the following:

"To measure this displacement we generated a series of centroids by calculating the average position of α-carbon atoms for residues 65-73 to represent the α0 helix in each protomer. In the CJF-III-288 structure, the α0 helix is displaced approximately 7.8 Å, 1.5 Å and 1.3 Å (for protomers A, B and C, respectively) away from corresponding α0 helix in the BNM-III-170 structure (Fig. 8b). We also created centroid to represent gp41 center by calculating the average of the α-carbon positions of the central gp41 α7 helices, residues 570-595 from the three protomers. Using the gp41 center as a hinge point, we measured α0 helix angular displacement in the superposed CJF-III-288-BG505 SOSIP.664-17b and BNM-III-170-BG505 SOSIP.664-17b complexes. The angular displacements were 11°, 1.6°, 0.1° in protomers A, B and C respectively."

• The authors present several measurements based on the structures to argue that the 17b Fab binding orientation has shifted in the CFJ-III-228 liganded structure compared to the BNM-III-170 bound structure. It is not entirely clear whether the authors mean that the epitope is presented in a different orientation or if they mean that the 17b is bound in a different orientation. The former follows from the protomer position shifts the authors have established based on alignments and distance measures (if the epitope changes position the paratope changes with it if the bound state remains the same). The authors measure the 17b binding angle of approach based on two positions, the 17b centroid and gp41. As

above, a distance measure cannot be described as an angular term. Second, if the authors mean to indicate that the Fab binding orientation has changed, by measuring 17b orientation between the immobile gp41 and mobile g120, the measures will differ regardless of whether 17b positioning has shifted since it has already been established that gp120 has shifted. These measures must use gp120 and 17b points of reference to determine whether the 17b binding angle has changed. If this is not the meaning, the language requires clarification. The map fits for 17b cannot be assessed based on the figures provided but issues in the cartoon representations in extended data figure 5a suggest that the fits are poor for at least the cyan colored structure. This presumably corresponds to the lowest resolution Fab visible in panel c of extended data figure 4. At the contour presented in this figure, each antibody appears to be somewhat poorly resolved. A figure showing the fits of the entire Fab and at the paratope are needed. These are especially critical for evaluating the claim that the 17b binding orientation has shifted. Finally, considering the poor Fab resolution as the distance from the paratope increases, the authors should consider measuring using atoms that are well resolved in the Fab and the antigen to mitigate issues related to uncertain fits. If the Fab orientation has indeed shifted, this should be evident in changes to the interaction at the binding interface.

Response: We thank the reviewer for raising this. We do not observe any changes to the 17b epitope or its angle of approach to any of the three gp120 protomers within the Env trimer. These interactions are essentially identical to those observed in other Env-17b complex structures, including the BNM-III-170-BG505 SOSIP.664-17b Fab complexes used here for comparison. Additionally, we do not detect significant differences in the quality of densities within the 17b binding interface on the trimer protomers in our cryo-EM structure. To support this, we generated Supplementary Fig 7, which is now included in the paper. This figure displays the experimental densities of the 17b Fab variable region bound to gp120, along with a superimposition of gp120-17bb Fab protomers from our CJF-III-288-BG505 SOSIP.664-17b complex and those of the BNM-III-170-BG505 SOSIP.664-17b complex.

The changes in the 17b angle of approach refer to its angle of approach to the Env trimer as a whole, rather than to individual gp120 protomers. These differences arise due to changes in the orientation of gp120 relative to the gp41 helices in the trimer. The description in the text has been updated as follows (see lines 355-373 of the "track-change" version of the manuscript):

"To quantify the differences in the angles of approach of the Fabs for each trimer protomer between the two complexes, we used the gp41 center as the hinge point and the centers of the Fabs to measure the angular displacements (Fig. 8 a,b). The differences we measured vary for each gp120 protomer with angular 17b displacements for protomers A, B, and C of 10.4°, 1.5°, and 1°, respectively. It should be noted that the observed differences in the angle of 17b's approach occurs only in the context of the Env trimer as a whole and is the result of changes in the orientation of gp120 relative to the gp41 helices within the trimer. No changes were observed in the 17b epitope or its angle of approach to any of the three individual gp120 protomers when these were analyzed individually. The interactions of 17b Fab with gp120 are essentially identical to those observed in other Env-17b complex structures, including the BNM-III-170-BG505 SOSIP.664-17b complexes used here for comparison (Supplementary Fig. 7). The individual gp120-17b variable region dimers from both complexes can be superimposed with an RMSD value of 1.96 for

main chain atoms. Thus, the changes in the angle of approach of 17b to the Env trimer we describe here results solely from changes in the orientations of the gp120 protomers relative to the trimer gp41. This change is most prominent in protomer A, which includes a significant displacement in the relative orientation of the variable heavy and light chains of 17b for that protomer. (Fig. 8b).”

• The claims regarding 17b orientation shifts in the cryo-ET are not convincing at the resolution of these experiments unless, again, the meaning is that 17b binding follows gp120 rotations. The fit uncertainty for the models cannot resolve whether 17b has shifted position on gp120 or if the 17b position differs due to gp120 rotations about the gp41 base. The map features between the two different liganded structures are sufficient to establish the the overall rotation of a protomer-Fab complex differs, but this finer grained analysis is not supported. Together, the results are more supportive of gp120 shifting position and therefore presenting the epitope in a differing position. An unchanged 17b binding orientation results in the 17b density following the gp120 angular shift. If the authors believe this is incorrect, more careful measurement and high confidence fits in the regions used in the measurements need to be presented.

Response: As suggested by the reviewer and clarified above, “17b binding follows gp120 rotations”.

• The scale of the numbers identify the smFRET states in figure 5 a and b should be clarified. Is the scaling identical to the relative differences in state occupancy? Preferably, the number sizes would not differ so that the reader can assess the relative differences based on the data. Numerical differences provided elsewhere can be used to examine this more carefully. Figure 5e warrants further discussion in the results section. While there is a general trend toward greater ADCC activity with changing state occupancy, a clear asymptote is visible in the data over which a wide range of ADCC activities is observed despite similar conformational distributions. This suggests an additional factor that is not measured in the smFRET affects the ADCC activity.

Response: Based on the reviewer’s comment, we have now standardized the number scaling for each state occupancy in the new Figure 6. The wide range of ADCC observed, despite similar conformational distributions, could be attributed to varying levels of other classes of CD4i nnAbs present in these plasma samples. Some of these antibodies also target epitopes exposed in State-3, thereby contributing to the observed ADCC response. This point is now addressed in the result sections of the revised manuscript at lines 261-265 of the “track-change” version of the manuscript.

Reviewer #4 (Remarks to the Author):

The manuscript submitted by Richard et al. is a detailed analysis on the working action of novel compounds for antiviral therapy against HIV. The focus is on antibody-dependent cellular cytotoxicity (ADCC) which represents one of the primary effector mechanisms used by the immune system to clear infected cells. HIV-1 envelope glycoprotein (Env) trimer represents the only viral protein exposed on the surface of HIV-1-infected cells and therefore serves as the primary target for ADCC-mediating antibodies. The conformational landscape of Env is described as dynamic, where multiple states from “closed” to “fully open” are available and their population plays a key role for the action of antiviral compounds, e.g. the closed conformation is resistant to non-neutralizing antibodies. The paper shows that a newly developed compound CJF-III-288, in combination with anti-CoRBS Abs, potently stabilizes an asymmetric open conformation of Env using a combination of biochemical and biophysical experiments.

Since I am not an expert in HIV cell biology nor the structural biology of the involved protein complexes, my review focusses on the assessment on the quality of the smFRET data and their embedment into the paper. I found the article very hard to read (for the non HIV-expert) and to orient myself. In general, the involved groups have already published smFRET papers of HIV proteins which are (too) briefly summarized in the introduction. Thus, for me a comprehensive figure in the introduction with a summary on the key aspects of Env conformations would help tremendously to better understand the findings the paper, how the assay was set up etc.

The paper might have the potential for Nature Communications, yet it requires revisions and an additional expert assessment as to whether the research questions themselves have the relevance to be suitable for the journal. My comments regarding the FRET data are:

Response: We thank the reviewer for her/his assessment of our manuscript.

- The assignment of the states 1-4 is for me (without further information) arbitrary since it shows a continuum of all available FRET efficiency values; see Figure 5a/b

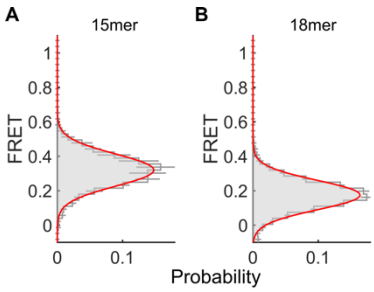
Response: The Reviewer fairly notes that we did not provide sufficient information on the analysis of our smFRET data. The four FRET states were not identified from fitting the FRET histograms shown in Figure 6a-b to Gaussian distributions. Indeed, as the Reviewer aptly points out, this would likely lead to identification of arbitrary states given the width (ie, standard deviation) of each Gaussian and the breadth of the FRET distribution. Instead, the four FRET states were identified from hidden Markov modeling (HMM) of individual smFRET trajectories. This analysis identified four discrete—yet inherently noisy—FRET states, which were used to construct the Gaussian distributions in Figure 6a-b. The inherent noise of each state gives rise to the width of each Gaussian. The results of this modeling was previously published in Alsahafi et al., Cell Host Microbe 2019 (PMID: 30974085). Furthermore, past publications have reported biological control experiments, such introduction of receptors and antibodies, that stabilize each of the four states. Most important in this regard were Munro et al., Science 2014 (PMID: 25298114); and Alsahafi et al.,

Cell Host Microbe 2019 (PMID: 30974085). Further information on the analysis of smFRET trajectories is provided in the Online Methods.

- Please determine the photon-limited width of a static inter-dye distribution to allow a clear relation of the data and the fitting model; e.g., currently the distribution of state 1 (and others) could also be related to >1 states; an unbiased approach for selecting the total number of states should be presented

Response: As noted above, discrete FRET states with inherent noise were identified using HMM analysis. Given the roughness of the energy landscape governing Env conformational dynamics, additional state might arise if data were acquired at higher time resolution. However, at present, limitations in the signal-to-noise ratio of the smFRET data preclude us from acquiring data faster. We are always working to improve our assay and increase the resolution of our measurements, but we prefer not to speculate in the text about the potential existence of additional states.

To address the Reviewer's question regarding the photon-limited width of a FRET histogram, we present data in Rebuttal Fig. 2 acquired from double-stranded DNA oligonucleotides labeled at either end with the same donor and acceptor fluorophores as used in the Env experiments. The distributions are slightly narrower than seen for Env, which reflects increased noise in the Env data due to fluorophore interactions with microenvironment and/or local flexibility of the fluorophore attachment site. Future studies in which technical advances are made to the smFRET assay, including perhaps upgraded instrumentation, will assess whether additional conformational states can be resolved.



Rebuttal Fig. 2. FRET histograms from (**A**) 15-mer and (**B**) 18-mer DNA oligonucleotides labeled at opposite ends with donor and acceptor fluorophores.

- Provide expected distances based on accessible volume calculations for inter dye distances in the proposed states and compare to experimental results

Response: This issue was partially addressed in a previous publication, Lu et al., Nature 2019 (PMID: 30971821). In this previous study, we used molecular dynamics simulations to estimate the space that the fluorophores sample using the available atomic coordinates of Env in the State 3 conformation, which was in the range of 60-75Å, in agreement with the 0.45-FRET state. This predicts an R0 of approximately 60Å, which is in agreement with our studies of other viral glycoproteins labeled with the same fluorophores. Unfortunately, atomic resolution structural data is not available for the other observed Env conformations. Therefore, we do not have sufficient structural data to perform this analysis for the other conformations.

- What are (were in previous publications) the effects of surface immobilization

Response: Unfortunately, we are not able to test the ability of a virus to enter cells after the virus has been surface immobilized. However, over several publications (PMID: 25298114; PMID: 27795397; PMID: 29561264; PMID: 30971821; PMID: 30974085) we have seen that the conformation of Env, as visualized in this smFRET assay, responds to mutation and biologically relevant ligands in predictable ways. This provides confidence that Env is not interacting with the quartz surface in a way that is affecting its ability to engage with ligands. Furthermore, the 150-nm virion is immobilized by way of a biotinylate phospholipid. The phospholipid contains a 2-kilodalton PEG spacer between the viral membrane and the streptavidin-coated quartz surface. In this way, the assay is setup to minimize interaction between Env and the surface.

- Do immobilization and dye attachement cause changes in the function of Env, how can this be characterized and excluded?

Response: As stated above, we cannot test the ability of a virus to enter cells after the virus has been surface immobilized. However, we have tested over the course of several publications (PMID: 25298114; PMID: 27795397; PMID: 29561264; PMID: 30971821; PMID: 30974085) the ability of Env on a surface immobilized virion to bind receptors and antibodies. These ligands are specific to native Env tertiary structure, and induce conformational changes in Env that are consistent with their function in virological assays. These experiments have been validated with bulk binding assays, and infectivity and neutralization assays. We are therefore confident that immobilization and fluorophore attachment are not significantly impacting Env structure and function.

- Are the results obtained by smFRET independent of the used dye pair? Please provide controls.

Response: We are at present limited to performing our smFRET experiments with Cy3 and Cy5, and derivatives thereof, such as LD650. This limitation is partly a result of the lasers and microscope optics that we currently have access to. Our efforts to use fluorophores from alternative sources that have similar spectral properties (e.g. Alexa fluors or Atto dyes) have failed to provide data of sufficient quality, owing to their relatively low brightness and photostability. At present, unfortunately, we are unable to collect smFRET data with alternative fluorophore pairs without making significant investments into our instrumentation.

Reviewer #1:

I am satisfied with the response.

Response: We thank the reviewer for her/his positive assessment of our manuscript.

Reviewer #2

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We thank the reviewer for her/his positive assessment of our manuscript.

Reviewer #3 :

The author's updates are sufficient. Lines 353-366 are, however, verbose. Stating measures of the binding interface indicate no change in 17b contact is sufficient to support the claim that the angle of approach differs due to gp120 interface presentation.

Response: We thank the reviewer for her/his positive assessment of our manuscript. Based on reviewer comment, we have revised this section to remove redundant explanations

We took the opportunity to make minor changes to the Results, Methods, and Figure Legends (Figure 9 e,f,g) to clarify this interpretation

We have added the gp120 and gp41 protomers from the BNM-III-170 structure (PDB: 7LO6) into Fig. 9e (light green/grey) to clarify that the difference in the 17b Fab positions coincides with the gp120 protomer displacement.

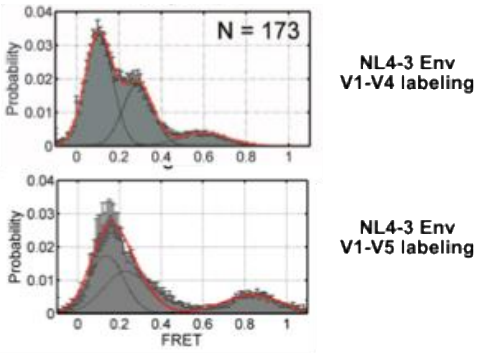
Reviewer #4 (Remarks to the Author):

On my part of the questions no significant improvement was done during the revisions and the questions were also not answered in a satisfying way. The paper should in my opinion not be published in its current form.

- identification of states: it is unclear and without the use of a second pair of fluorophores (which could be of the same spectral characteristics, e.g., rhodamine fluorophores) it is not clear to me if any of the smFRET data make sense

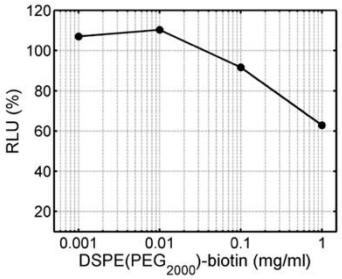
Response: Our smFRET approach has been extensively validated over the past decade in numerous peer-reviewed publications from our group and others—including Science, Nature, Cell Host and Microbe, etc. This approach has also been expanded beyond HIV-1 Env, to influenza HA, SARS-CoV-2 Spike, Ebola GP, etc. The findings are consistently corroborated by structural and functional approaches. smFRET is now widely recognized as a gold standard method for analyzing envelope glycoprotein conformational dynamics. We were therefore surprised by the reviewer's continued concerns regarding the assignment of Env conformational states and the validity of the smFRET data. Here, we summarize the published data that validates the interpretation of our smFRET data:

- **In the initial presentation of smFRET applied to HIV Env, data were collected from two distinct perspectives—labeling pairs on V1 and V4 loops, or V1 and V5 loops—and on two different strains. All data yielded internally consistent results, which argues against artifacts related to fluorophore attachment sites (Rebuttal Figure 1, from Munro et al. Science 2014. PMID: 25298114).**



Rebuttal Fig. 1. smFRET data acquired on HIV-1 Env from two distinct perspectives, as indicated. Both labeling schemes lead to identification of three states.

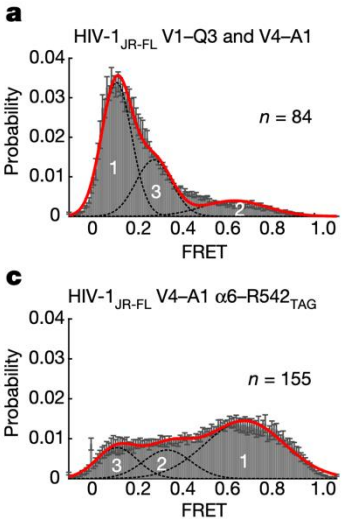
- **Also in the initial publication, the impact of incorporation of the synthetic biotin-phospholipid into the viral membrane, which enables surface-immobilization was assessed using pseudovirion infectivity. No loss of infectivity was detected across the range of biotin-phospholipid concentrations needed for efficient immobilization (Rebuttal Figure 2, from Munro et al. Science 2014. PMID: 25298114).**



Rebuttal Fig. 2. Infectivity (RLU) of HIV-1 virions incubated with varying concentrations of DSPE(PEG_{2000})-biotin. 0.01 mg/ml is used during preparation of samples for smFRET imaging.

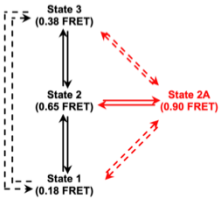
- **In a follow-up publication, mutagenesis of the CD4-binding site was used to verify the structural nature of States 2 and 3 (Ma et al. eLife 2018. PMID: 29561264).**

- **In a study of Env-based immunogens, we collected data from two perspectives, one which was entirely novel (V4 and R542 in gp41), and on two additional strains. In the V4 – R542 perspective, the FRET values were inverted, meaning the low FRET state in the V1-V4 perspective became a high FRET state in V4-R542; and the CD4 bound state, a high FRET in the V1-V4 perspective, became a low FRET state. Importantly, in a time course of activation, Env transitioned from the ground state all the way to the CD4-bound state. Thus, data were internally consistent, providing further evidence of the robustness of the approach to variation in fluorophore position (Rebuttal Figure 3 from Lu et al. Nature 2019. PMID: 30971821).**

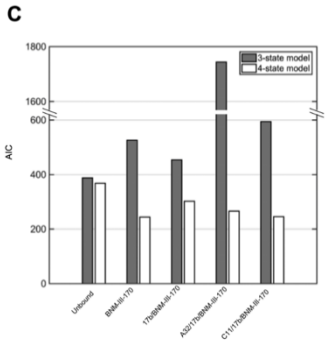


Rebuttal Fig. 3. smFRET data acquired on HIV-1JR-FL from two distinct perspectives, as indicated. Here again, both labelling schemes led to the identification of three states.

- **We conducted a quantitative analysis of hidden Markov model fitness using maximum likelihood optimization and the Akaike information criterion. We found that for unbound HIV Env, three FRET states was sufficient to represent the data. Upon addition of ligands, a model with four FRET states provided a better fit (Rebuttal Figure 4, from Alsahafi et al. Cell Host Microbe 2019. PMID: 30974085).**



- **Several publications have investigated the mechanisms of inhibition by antibodies and CD4-mimetic compounds in terms of their impact on Env conformation. In all cases, smFRET imaging in the presence of ligands led to observations of the same FRET states but with shifted occupancies. These publications provide solid evidence that smFRET imaging is reporting on functionally relevant Env conformations (Munro et al. Science 2014. PMID: 25298114; Lu et al. Nature 2019. PMID: 30971821; Alsahafi et al. Cell Host Microbe 2019. PMID: 30974085; Marchitto et al. J Virology 2024. PMID : 39248460).**



Rebuttal Fig. 4. Evaluation of model fitness using maximum likelihood estimation and the Akaike information criterium (AIC). Three- and four-state models were considered. In the presence of CD4mc and mAb 17b, the four-state model provides better fitness (lower AIC).

- **The reviewer seems to express concern about the specific fluorophores being used. The reviewer has suggested collecting data with alternative fluorophores, specifically rhodamine derivatives such as Atto dyes, which are spectrally similar to our preferred cyanine derivatives. Unfortunately, to our knowledge, Atto dyes conjugated to coenzyme A, which are required for our labeling strategy, are not commercially available. At present, we do not have the instrumentation necessary to synthesize our own**

fluorophores. Because of the sophisticated fluorescent labeling strategies that we utilize, we have limited ability to use alternative fluorophores. We would like to highlight that the fluorophores applied in this study are widely used by the smFRET imaging community. We would like to also note that we have repeatedly swapped donor and acceptor fluorophores with identical results. Finally, in the earlier applications of smFRET imaging to HIV Env (Munro et al. Science 2014. PMID: 25298114; Lu et al. Nature 2019. PMID: 30971821; Alsahafi et al. Cell Host Microbe 2019. PMID: 30974085), an alternative labeling strategy was used, which involved different fluorophores (Cy3B and Alexa647). As noted above, these earlier data are consistent with the data presented in the current manuscript, in we use Cy3 and LD650.

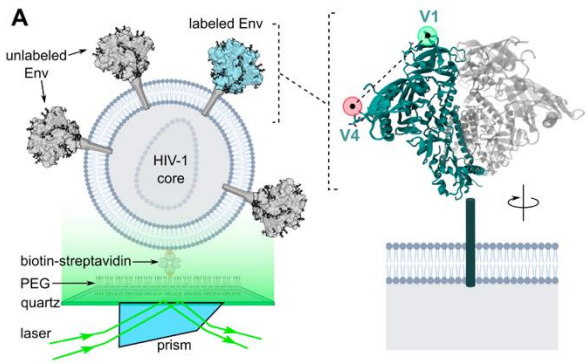
To address Reviewer #4's concern, we now present an HMM analysis of our smFRET data in which we repeat an earlier analysis to identify the number of FRET states. Through maximum likelihood optimization an application of the Akaike information criterium, we confirm that a 5-state model (4 non-zero FRET states, and a 0-FRET state) represent the data well. We also provide an analysis using an alternative HMM approach based on Bayesian inference (ebFRET). The two methods resulted in essentially indistinguishable results. This analysis supports the conclusion that a model with four discrete non-zero FRET states best explain the smFRET trajectories (see Supplementary Figure 5 and Methods section)

Importantly, we would like to clarify that while the smFRET data provide additional support for our conclusions, they are not central to the core findings of the study. The primary evidence supporting our conclusions comes from cryo-EM, cryo-ET, and functional assays. The smFRET data serve as a complementary approach that reinforces our observations that CJF-III-288, in combination with anti-CoRBS antibodies, stabilizes a more "open" Env conformation that improve the ADCC activity of anti-CoRBS.

- functional controls are needed: the authors state in their response: "We are therefore confident that immobilization and fluorophore attachment are not significantly impacting Env structure and function." Please provide concrete numbers (e.g., affinities +/- dye) and other concrete evidence

Response: We have previously tested the effect of fluorescent labeling on influenza HA and Ebola GP and found minimal impact (Das et al. Cell 2018. PMID: 29961575; Das et al. PLoS Biology 2020. PMID: 32040508.) Nonetheless, we acknowledge that evaluating the impact of fluorophore labeling on Env function is a worthwhile control. Accordingly, we are now providing evidence that Env labeling has no measurable effect on infectivity (see Supplementary Figure 4). However, testing the functionality of Env on virions that are surface immobilized for our smFRET assays is not technically feasible. Virions are immobilized within sealed quartz microfluidic chambers that are not amenable to cell culture. The virions are immobilized on

streptavidin-PEG-coated surfaces through incorporation of a biotin-PEG$_{5000}$-phospholipid into the viral membrane (see Rebuttal Figure 5). We have verified (see above, Rebuttal Figure 2) that the phospholipid itself does not affect virus infectivity. Given that the Envs being imaged are incorporated in native virions, which separate Env from the quartz surface, we consider it highly unlikely that the Envs are interacting significantly with the surface.



Rebuttal Fig. 5. Experimental setup for smFRET imaging of HIV-1 virions using TIRF microscopy.

We took the opportunity to make other changes in the manuscript:

- **The methods section for cryoET sample preparation was expanded to provide more information about production and purification of the HIV-1$_{BaL}$ virus used for cryoET. Acknowledgements were added for the individuals who provided this virus.:**

  "We thank Julian Bess, Jr and Jeff Lifson of the AIDS and Cancer Virus Program of the Frederick National Laboratory for Cancer Research for providing the SupT1 HIV-1 BAL/A66-R5 CL.1 CLN445 cell line produced virus (lot P4338) used in this study. Production of the virus was supported with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. 75N91019D00024/HHSN261201500003I"

- **We have slightly revised the Env tilt angle calculation to include a wider angular search range on the membrane refinement. Previously, the search range was restricted to ~30-40 degrees. After allowing a wider search range (~60 degrees), we see a longer tail towards the higher angles of the distribution and a right-skewed distribution shape. The median and interquartile range of this distribution are nearly identical with the previous results and still closely correspond to observations from class averages.**

  To further support these calculations, we have included Supplementary Fig. 11. This figure describes the tilt angle calculation (a) and shows central slices of the density map before and after membrane refinement (b). We also provide individual tomographic slices of trimers corresponding to calculated angles along the distribution (c) and provide an example of the calculated perpendicular membrane vectors plotted with the final structure mapped back to the tomogram (d). We believe this additional figure adds confidence to the measurements and will improve reproducibility.

- **We made the following addition in the Acknowledgements section:**

  "Placental cord blood for humanized mouse engraftment was provided by the Yale University Reproductive Sciences (YURS) Biobank"