nature portfolio

Peer Review File

Enhanced and sustained biodistribution of HIV-1 neutralizing antibody VRC01LS in human genital and rectal mucosa

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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)
In their manuscript, Maria Lemos, Rena Astronomo, and colleagues investigate the distribution of HIV-1 broadly neutralizing antibody (bNAb) variants VRC01 and VRC01LS in mucosal tissues and rectogenital secretions after a single intravenous infusion in HIV-negative individuals. Distribution of bNAbs into these compartments may be critical for effective antibody-mediated prevention of HIV transmission through sexual exposure. Given the preclinical evidence for the importance of differences in mucosal antibody levels in antibody efficacy, the clinical data on the direct comparison of VRC01 and VRC01LS provided in this manuscript will be informative for the field of HIV antibodies and antiinfective antibodies in general.

The manuscript is overall well written and straightforward, the data is generally presented clearly, and the discussion and conclusions appear sound. Some of the analyses are limited by the relatively small number of participants and/or evaluable samples (e.g., rectal secretions).

Main comments

1)
Antibody levels are provided in a weight-adjusted manner (µg/ml per kg body weight). This appears odd because weight-adjustment already occurred at the step of antibody dosing: 30 mg/kg (i.e., the total antibody dose administered is dependent on the individual participant's body weight). It also makes comparisons of antibody levels to previously reported data more difficult because serum concentrations for HIV bNAb PK studies have typically been provided in µg/ml (absolute concentration). Finally, for the immunohistochemistry analyses shown in Figures 5 and 6, absolute intensities that do not take body weights into account are analyzed (i.e., the use of weight adjustment is inconsistent). The rationale provided for doing weight-adjusted is the body weight difference between male participants receiving VRC01 and VRC01LS (line 106). However, as indicated above, the absolute administered dose takes these differences into account and there may well be other influencing parameters than just body weight (e.g., BMI, etc.). I would suggest to present the data for the main analysis in a more traditional absolute way (i.e., µg/mL) and provide weight-adjusted data as a supplement.

2)
Table S3: Adverse events should be listed in more detail (type of AE, grading, relatedness for individual AEs), as these were primary endpoints of the trial according to the protocol. It would also be helpful if the reactogenicity event observation period (3 days after infusion according to methods section) could be explicitly stated in Tables S1 and S2.

Minor comments

3)
One of the participants is reported as having tier 2-detectable ADAs. Do the PK parameters for this individual deviated from the overall trend? Supplementary Table 4 states (in the foot note) that this participant was negative for tier 3 testing and it may be worthwhile to include this information in the manuscript text.

4)
One of the participants only received 60% of the planned dose but has been included in the analyses (line 328). It may be helpful to indicate this person by a dedicated symbol in Figure 2.

5)
Figure 1A: From the figure and legend, it is not immediately clear to which comparison the p values relate (perhaps include in legend).

6)
Figure S1: According to the legends, “Points away from red dashed lines (perfect concordance) indicate that Singulex estimates were conservative compared to ELISA.”. However, as almost all points are above the dashed line, doesn’t it rather suggest the opposite (i.e., antibody levels determined by the Singulex assay were rather higher (which is less conservative to me) than antibody levels determined by ELISA)?

7)
Figure S3: Data for VRC01 and VRC01-LS are display in the same plots (which is different from Fig. S4) and only data for VRC01 are included in the correlation analyses. While this can be deduced from the figure legend, I would suggest to a) indicate VRC01 and VRC01-LS data points with different colors as in S4, and b) make clear in the figure itself, that the correlation results only pertain to VRC01 to avoid confusion.

8)
Lines 38-43: For the uninformed reader, the summary of the AMP trials reads overly positive. Overall, the trial failed to demonstrate prevention efficacy and it could be made clearer that VRC01 was not sufficiently active against the majority of the strains the AMP trial participants appear to have been exposed to. The likely need for using antibody combinations could also be included.

9)
Lines 43-44: The “200-fold above” statement refers to the 80% neutralization titer (the number should be included).

Typos:

10)
In Figure 1A, the box for the VRC01-LS infusion at week 0 should say “30 mg/kg” as dose, not “3 mg/kg”.

11)
Line 105: It should say “VRC01”, not “VR01”.

Reviewer #2

(Remarks to the Author)
Overall, an excellent manuscript reporting a significant translational human study.

Comments

Abstract, L31: no justification is given for the use of ‘higher’. I appreciate the comparisons between the two Mabs are complex, but would the authors like to consider a more definitive statistical statement?

P6, L110-112: I see one VRC01 recipient had an infusion reaction, which was classified as moderate. The clinical features sound a little worrying for the medics on site. Could we be re-assured in the text that the reaction subsided promptly without any treatment? The publication Takuva S (2022) doi: 10.1097/QAI.0000000000002892 could be used to illustrate the frequency and spectrum of these reactions.

P9, L194-195: The text “suggesting that rectal tissue may contain some protein-rich areas that do not efficiently retain both mAbs” sounds a little mysterious to me and perhaps speculative. Could the authors either clarify or modify?

P10/11, L228-236, & Fig 5, panels B & D: Firstly, I agree that the “variable patterns … make manual scoring difficult”. But in reference to Figure 5 I think the authors may be using the term ‘stratum corneum’ incorrectly. Ectocervical and vaginal epithelial cell layers are usually classified into 4 (or sometimes 3, or occasionally 5) layers, and referred to, in ascending order, as basal, parabasal (sometimes called spinous), intermediate (sometimes called stratum granulosum), and superficial (also known as stratum corneum). The epithelial staining in Figure 5B (both mAbs) is clearly in the intermediate (granular layer, cells with glycogen inclusions) layer, not in the stratum corneum / superficial layer. The staining in 5D (VRC01LS) is a little more complex, with strong staining in the intermediate / granular layer. There may be a little staining in the most superficial layer of the 50 um panel, but inspecting the parent 500 um panel the superficial staining looks non-specific. If the authors accept this point of correction, they will need to alter the text on P13, L292-293. I am also not convinced by the claim and illustration of differences in Figure 5 between cytoplasmic [C] vs pericellular [P] mAb localization although I do not have the benefit of in vivo high power microscopic examination.

P12, L261: The authors need to explain how they arrived at the estimate that “VRC01LS concentrations last >1 year”.

P13, L291-2: “The greater distribution of VRC01LS suggests that FcRN aids distribution in cervico-vaginal epithelium”. This statement is at odds with the authors assertion on P12, L275-6 that mAb distribution within the cervico-vaginal epithelium is principally by convection, and not through FcRn-mediated transport, which I support.

P14, L307-308: The authors state that they could not demonstrate differences between the mAbs for penetration into seminal plasma, although the half-lives are clearly different (Figure 2B, lowest panel) with p=0.067 on a small sample size, and despite their prior claims on P12, L260-261.

P4, L61, & P34, Ref 21: There are doubts about the reproducibility of this work. Our own group was unable to confirm these murine findings, and the claimed phenomenon of FcRn-mediated bi-directional cervico-vaginal IgG transport clearly does not occur in humans.

Reviewer #3

(Remarks to the Author)
The manuscript ‘Fc-modified, HIV-1 broadly neutralizing monoclonal antibody, VRC01LS, shows enhanced biodistribution in human genital and rectal mucosa compared to VRC01 in a randomized clinical trial’ by Lemos, et al presents a novel longitudinal characterization of the biodistribution and localization to mucosal sites of HIV acquisition of two clinically relevant broadly neutralizing antibodies (bNAbs) following single-dose passive immunization of men and women. The manuscript, from a top-notch group of investigators, clearly demonstrates a primary important finding -- that the LS modification confers approximately 3-fold enhancements to bNAb Cmax and half-life in vaginal, cervical and rectal tissues, resulting in VRC01LS persisting >1 year at these relevant sites of HIV acquisition. As such, these results extend earlier reports of VRC01/VRC01LS pharmacokinetics in serum and have obvious relevance toward understanding bNAb-mediated protection at sites of HIV exposure.

In addition, the data presented raise interesting points concerning apparent underestimation of mucosal tissue-associated bNAb levels inferred from bNAb levels measured in mucosal secretions, as well as the relative paucity of bNAb within rectal glandular epithelium vs cervicovaginal eptithelium.

The authors duly note some of their studies’ limitations, including the relatively small number of study participants and lack of female rectal and male genital tissues for analysis. Overall, the paper is very clearly written and the methods pleasantly contain sufficient detail.

A few suggestions that may improve the readability of the manuscript are:
1. Improve the resolution of the IHC panels presented in Figure 4A.
2. Present isotype-stained control sections at high magnification, corresponding to Figure 4 panels B-E
3. To the main text, add a brief description regarding how manual scoring was performed for rectal immunohistochemistry presented in Figure 4F and a description/reference(s) to the use of H-scores regarding cervical/vaginal analyses in Figure 5E

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for taking my comments and suggestions into consideration. I feel that most aspects have been adequately addressed in the authors’ response and the revised version of the manuscript.

A small number of remaining comments:

1)
In the added Fig. S1C, serum concentrations of VRC01 and VRC01-LS are now presented in “µg/mL”. In Fig. 2A, they are displayed as “µg/ml per kg”.

Comparing the values in the individual graphs, something seems to be off. For example, VRC01-LS concentrations in males at week 13-14 appear to be essentially identical (ca. 1) in both the “µg/ml per kg” (Fig. 2A) and “µg/ml” units (Fig. S1C) – which I believe cannot be correct. I suggest double-checking values, curves, and axis labels.

Moreover, the absolute values written out (“median cmax”) should match the graph units. For example, in Fig. 2A, the median cmax (determined at wk 1-2) in males are given as 240.5 and 731.1 for VRC01 and VRC01-LS, respectively. However, these values are way above what is displayed in the graphs (cmaxes <10). In Fig. S1C, the median cmaxes in males are given as 0.241 and 727 for VRC01 and VRC01-LS, which would be a >1.000-fold difference. I suggest double-checking all numbers.

2)

The AE table (Table S3) has been updated but I feel it can have a bit more clarity.

The separation between related and unrelated AEs could be clearer. Below the heading “Participants with one or more AEs” only two participants are listed at first (presumably those with related AEs). Perhaps it would be sufficient to change the headings to “Participants with Study Drug-Related AEs”, “Type and Severity of Study-Drug Related AEs”, and “Participants with AEs not Considered Study-Drug Related”?

Moreover, I would suggest to rephrase the line “Number (n)” to “Participants (n)”. Currently it suggests that these numbers indicate the number of AEs as they are listed right under the heading “Study related and Unrelated Adverse Events”.

3)
In Figure S5 (previously Figure S3), the y-axis has been updated to indicate that only VRC01 levels are shown. If no VRC01-LS levels are included, then this should also be removed in the legend. In the sentence “Correlations [...] in male participants IV infused with VRC01 or VRC01LS [...]” that starts the legend, the “or VRC01LS” should be removed.

Reviewer #3

(Remarks to the Author)
Thank you for your manuscript revisions - all of my suggested changes have been incorporated.

Editorial note: this reviewer was additional asked to comment in place of reviewer 2 who was unavailable to provide comment at this stage. And stated "I feel that Reviewer #2’s concerns have been fully addressed in the revised manuscript."

We thank the reviewers for their thorough review of our manuscript. We provide below a point-by-point response to their comments as follows.

Reviewer #1 (Remarks to the Author):

In their manuscript, Maria Lemos, Rena Astronomo, and colleagues investigate the distribution of HIV-1 broadly neutralizing antibody (bNAb) variants VRC01 and VRC01LS in mucosal tissues and rectogenital secretions after a single intravenous infusion in HIV-negative individuals. Distribution of bNAbs into these compartments may be critical for effective antibody-mediated prevention of HIV transmission through sexual exposure. Given the preclinical evidence for the importance of differences in mucosal antibody levels in antibody efficacy, the clinical data on the direct comparison of VRC01 and VRC01LS provided in this manuscript will be informative for the field of HIV antibodies and antiinfective antibodies in general. The manuscript is overall well written and straightforward, the data is generally presented clearly, and the discussion and conclusions appear sound. Some of the analyses are limited by the relatively small number of participants and/or evaluable samples (e.g., rectal secretions).

Response: We thank the reviewer for highlighting the potential contribution of the manuscript to the field. We acknowledge the limitations of the small sample size for some sample types in the discussion (page #14).

1) Antibody levels are provided in a weight-adjusted manner (µg/ml per kg body weight). This appears odd because weight-adjustment already occurred at the step of antibody dosing: 30 mg/kg (i.e., the total antibody dose administered is dependent on the individual participant's body weight). It also makes comparisons of antibody levels to previously reported data more difficult because serum concentrations for HIV bNAb PK studies have typically been provided in µg/ml (absolute concentration). Finally, for the immunohistochemistry analyses shown in Figures 5 and 6, absolute intensities that do not take body weights into account are analyzed (i.e., the use of weight adjustment is inconsistent). The rationale provided for doing weight-adjusted is the body weight difference between male participants receiving VRC01 and VRC01LS (line 106). However, as indicated above, the absolute administered dose takes these differences into account and there may well be other influencing parameters than just body weight (e.g., BMI, etc.). I would suggest to present the data for the main analysis in a more traditional absolute way (i.e., µg/mL) and provide weight-adjusted data as a supplement.

Response: We did not sufficiently explain our rationale for body weight normalization of the antibody levels. This normalization was a deliberate decision balancing a few factors that we outline below.

- **a.** Normalization of antibody concentrations by body weight or dose is a common practice in drug PK analysis. It is especially useful when participants receive different doses (e.g., as shown in Figure 3 of Davda, *et al.,* mAbs 6(4):1094-1102 (DOI: 10.4161/mabs.29095)). In our study, doses were listed as 30 mg/Kg of weight, which means that the total dose received by a participant is 30 mg multiplied by their body weight. Thus, dividing once by body weight is a single step normalization. Normalization by body weight is equivalent to normalization by dose in our study because the VRC01 and VRC01LS groups received the same 30mg/Kg dosage.
- **b.** Normalization by body weight is especially applicable to tissues, as the volume and mass of many organs do not increase linearly with body weight in adults.
- **c.** In our study, male participants receiving VRC01LS had a median weight of 103.25 Kg, whereas VRC01 male participants had a median weight of 77.5 Kg (p=0.008; page 5 and the revised Figure 1B). Without normalization, readers could question whether the higher concentration in the LS

group was due to the LS modification, or due to the higher amount of antibodies administered as a direct result of having higher body weight.

d. When normalized for body weight, the concentrations for males and females are also easier to compare (Figure 2A).

However, to support the comparisons of serum concentrations to other manuscripts, we have added Supplementary Figure 1C that shows the absolute concentrations in µg/ml and a corresponding caption in the figure legend stating that "(C) Pharmacokinetic profiles of absolute VRC01 (pink) and VRC01LS (purple) serum concentrations (ug/ml) in the males and females enrolled in the study.". On page 7, we also added the following statement: "In addition, the absolute serum concentrations (Supplementary Figure 1C) and the weight-normalized concentrations (Figure 2A) were consistent with those observed in earlier studies of VRC01 and VRC01LS administered at 30 mg/kg".

The reviewer is correct in that we provided body weight normalized data for all readouts of Singulex VRC01 and VRC01LS estimates, which is a continuous variable with well-characterized precision and accuracy. However, we were hesitant to apply body weight normalization to the manual scoring depicted in Figure 4C, which describes 5 discrete intensities of brown DAB staining, ranging from 1-5. Body weight normalization would artificially create more variability among the intensity scores than what was measured. In Figure R1 below, the body weight normalized data is presented, which indicates the increased durability of the VRC01LS infusion compared to VRC01, when the adjustment takes place. We have clarified in the methods in page 30: "Manual scoring of the intensity of the rectal lamina propria was conducted by visually inspecting and scoring the intensity of DAB with discrete numbers ranging 0-5, blinded to group assignment, participant and visit number. No body weight adjustment was applied to this measurement to preserve the discrete characteristics of the manual score".

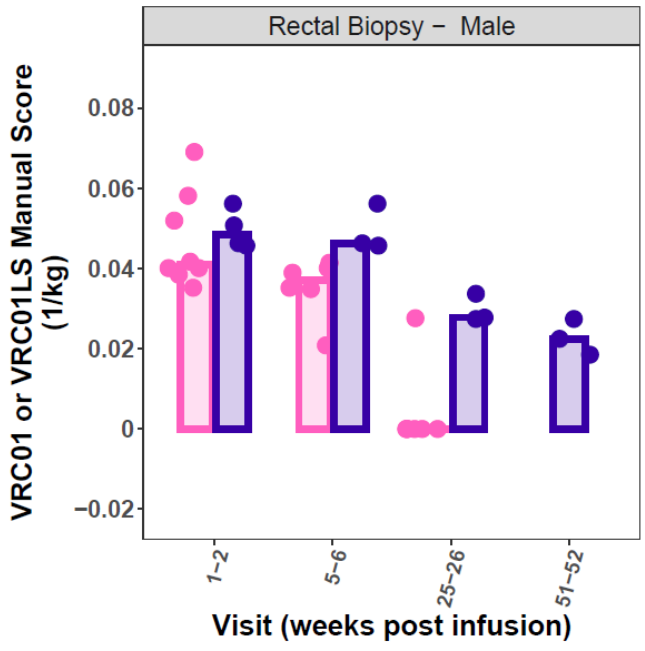

Figure R1. Body weight normalized manual scoring of the rectal immunohistochemistry using 5C9 for detection of VRC01 (pink) or VRC01 LS (blue) in males. Manual scores from 0 to 5 were divided by the participant body weight (Kg).

In the case of Figure 5, we presented the IHC quantification without normalization because the figure addressed only females, for whom no body weight differences were detected (Figure 1B). Since vaginal and cervical compartments are female-specific, comparisons to the normalized male groups were not necessary. However, we performed the statistical comparisons for cervical and vaginal H-scores both with

and without body weight adjustment and found similar results, except that the body-weight adjusted analysis also showed significant differences between VRC01 and VRC01LS groups in the vaginal epithelium (Figure R2).

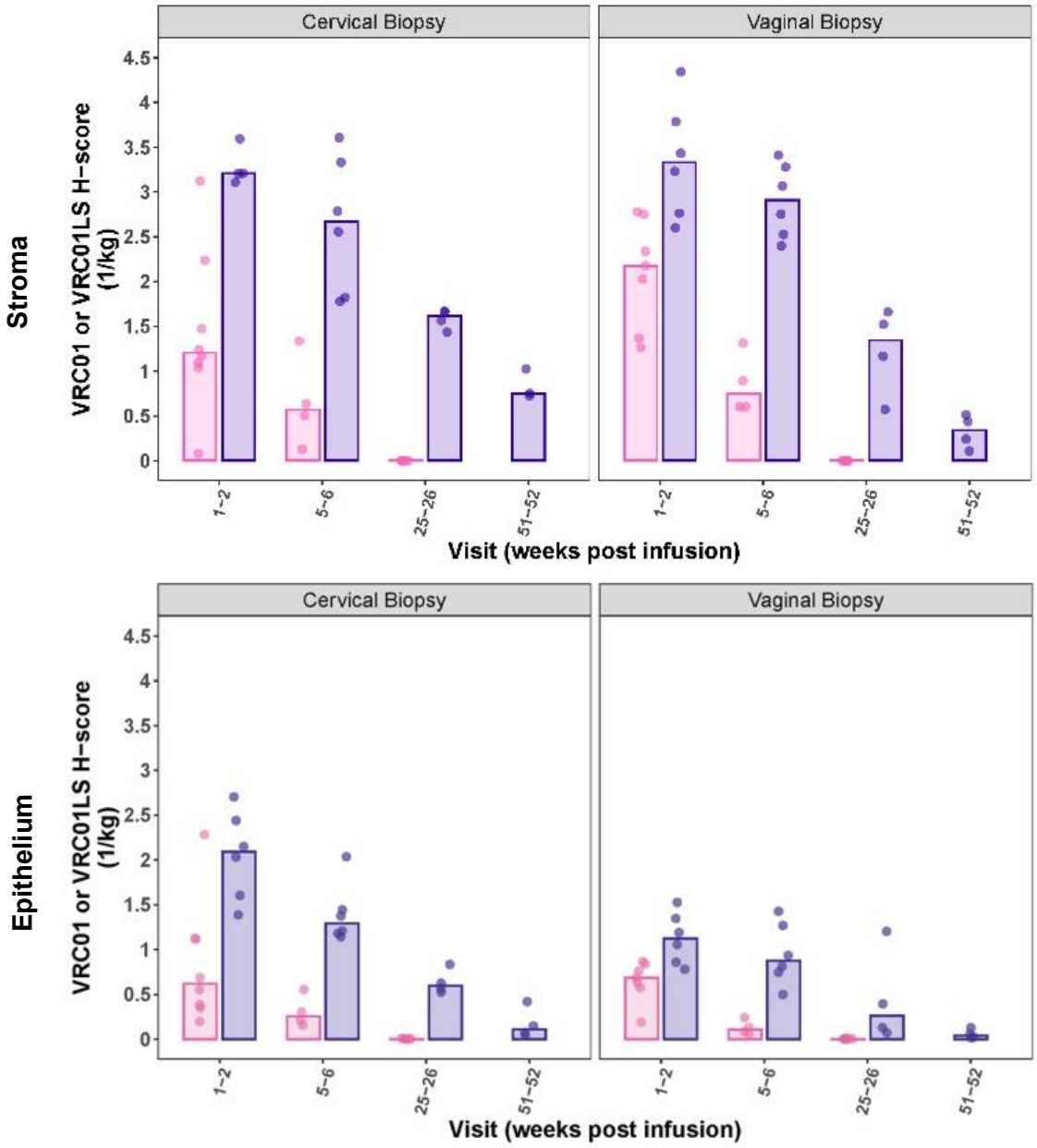

Figure R2. Body weight normalized H-scores of the cervical and vaginal stroma (top panels) and epithelium (bottom panels) by immunohistochemistry using 5C9 for detection of VRC01 (pink) or VRC01 LS (blue) among females. H-scores were divided by the participant body weight (kg). Comparisons between VRC01 and VRC01LS for cervical and vaginal stroma and cervical and vaginal epithelium were all $p<0.01$ at 1-2 weeks post-infusion.

2) Table S3: Adverse events should be listed in more detail (type of AE, grading, relatedness for individual AEs), as these were primary endpoints of the trial according to the protocol. It would also be

helpful if the reactogenicity event observation period (3 days after infusion according to methods section) could be explicitly stated in Tables S1 and S2.

Response: We have added the duration of reactogenicity to the Supplementary Tables 1 and 2. We have also modified the AE table to include the type and severity of the AEs, and the specific relatedness of the symptoms in Supplementary Table 3.

3) One of the participants is reported as having tier 2-detectable ADAs. Do the PK parameters for this individual deviated from the overall trend? Supplementary Table 4 states (in the foot note) that this participant was negative for tier 3 testing and it may be worthwhile to include this information in the manuscript text.

Response: As indicated in Supplementary Table 4, one participant had tier 2 ADA but not tier 3 ADA at baseline. We have now dedicated a paragraph to the ADA results (page 6) that states: " Tier 1 ADA antibodies were measured in all participants at the pre-infusion (baseline) visit and all participants reaching the last visit for each group (group 4 at 25-26 weeks, group 5 at 51-12 weeks). All but one infusion recipient tested negative for tier 1 antibodies at these timepoints. The VRC01 recipient with tier 1 positive antibodies, also tested positive for tier 2 but not tier 3 antibodies at baseline (Supplementary Table 4). This individual did not have any tier 2 antibodies at 25-26 weeks post-infusion and had no issues during the mAb administration."

At the request of the reviewer we have also added a statement on page 7 to indicate that there was no difference in the PK profiles of that participant compared to other participants in the VRC01 infusion group: "This intention-to-treat analysis included the participant who received a partial dose (Supplementary Figure 2) and the participant who had tier 2 ADA at baseline (Supplementary Figure 3); no significant differences were seen in their PK profiles compared to others in their infusion group." The PK profiles of this female participant are detailed in green in Supplementary Figure 3.

4) One of the participants only received 60% of the planned dose but has been included in the analyses (line 328). It may be helpful to indicate this person by a dedicated symbol in Figure 2.

Response: We found that a dedicated symbol was difficult to visualize in Figure 2 due to the quantity of data presented in the graphs. At the request of the reviewer, we have added a statement on page 7 to indicate that, as expected, the PK parameters of the participant did not deviate from the overall trend: "This intention to treat analysis included the participant who received a partial dose (Supplementary Figure 2) and the participant who had tier 2 (but not tier 3) ADA at baseline (Supplementary Figure 3); no significant differences were seen in their PK profiles compared to others in their infusion group". We have also added a new Supplementary Figure 2, which shows the participant who received the partial dose in green, to demonstrate this point in serum, rectal biopsies, rectal secretions and semen.

5) ***Figure 1A: From the figure and legend, it is not immediately clear to which comparison the p values relate (perhaps include in legend).***

Response: Thanks to the reviewer for pointing out this issue. We have updated Figure 1B to show the p-values comparing the age and body weight of VRC01 vs. VRC01LS recipients of both sexes assigned at birth.

6) Figure S1: According to the legends, "Points away from red dashed lines (perfect concordance) indicate that Singulex estimates were conservative compared to ELISA.". However, as almost all points are above the dashed line, doesn't it rather suggest the opposite (i.e., antibody levels determined by the

Singulex assay were rather higher (which is less conservative to me) than antibody levels determined by ELISA)?

Response: Thanks to the reviewer for pointing out this apparent inconsistency. It alerted us to an error on our part in Supplementary Figure 1. The axis labels were accidentally switched during figure assembly and are now corrected. The manuscript and interpretation have not changed, but the figure now matches the interpretation.

7) Figure S3: Data for VRC01 and VRC01-LS are display in the same plots (which is different from Fig. S4) and only data for VRC01 are included in the correlation analyses. While this can be deduced from the figure legend, I would suggest to a) indicate VRC01 and VRC01-LS data points with different colors as in S4, and b) make clear in the figure itself, that the correlation results only pertain to VRC01 to avoid confusion.

Response: Supplementary Figure 3 (now Supplementary Figure 5) shows only data from VRC01-infused male participants. For males, correlations for VRC01LS were not possible due to the small n. We agree with the reviewer that the axis labeling was confusing, and we have updated them to indicate this figure is showing data for VRC01 exclusively.

8) Lines 38-43: For the uninformed reader, the summary of the AMP trials reads overly positive. Overall, the trial failed to demonstrate prevention efficacy and it could be made clearer that VRC01 was not sufficiently active against the majority of the strains the AMP trial participants appear to have been exposed to. The likely need for using antibody combinations could also be included.

Response: We agree with the reviewer that our early reference to the AMP trials in the introduction could be misleading to an uninformed reader. Thus, we have made the following modifications to provide more clarity about the overall trial results and the data used to establish the proof-of-concept.

a) Lines 39-42: The sentence now reads "The demonstration in humans that the broadly neutralizing mAb, VRC01[1], could prevent acquisition of VRC01-neutralization sensitive HIV-1 strains in the Antibody Mediated Prevention (AMP) trials provides proof of concept for this approach, despite the lack of overall prevention efficacy in the trials."
b) Lines 44-45: "…protected against ~30% of circulating HIV-1 strains that were sensitive to neutralization by the mAb[3]"
c) Lines 47-49: We replaced the original sentence, "Thus, identification and evaluation of broadly neutralizing mAbs with even greater neutralization potency-breadth profiles are underway." with "Thus, to achieve these titers against most circulating strains, broadly neutralizing mAbs of greater potency and breadth are being identified and evaluated both alone and in combination."

9) Lines 43-44: The "200-fold above" statement refers to the 80% neutralization titer (the number should be included).

Response: The specific neutralization threshold (80%) has been added (line 45). The sentence now reads "Subsequent analyses estimated that sustained serum mAb concentrations 200-fold above the *in vitro* 80% neutralization concentration against the acquired viruses will be required to achieve 90% prevention efficacy".

Typos:

10) In Figure 1A, the box for the VRC01-LS infusion at week 0 should say "30 mg/kg" as dose, not "3 mg/kg".

Response: This typo in the figure has been corrected.

11) Line 105: It should say "VRC01", not "VR01".

Response: The typo has been corrected (line 121).

Reviewer #2 (Remarks to the Author):

Overall, an excellent manuscript reporting a significant translational human study.

1) Abstract, L31: no justification is given for the use of 'higher'. I appreciate the comparisons between the two Mabs are complex, but would the authors like to consider a more definitive statistical statement?

Response: Thanks to the reviewer for appreciating our work. We have modified the sentence and it now reads: "At 1-2 weeks, VRC01LS levels were ~3-4 times higher than VRC01 in serum (p=0.048), rectal (p=0.067), vaginal (p=0.003) and cervical tissues (p=0.003); these differences increased over time".

P6, L110-112: I see one VRC01 recipient had an infusion reaction, which was classified as moderate. The clinical features sound a little worrying for the medics on site. Could we be re-assured in the text that the reaction subsided promptly without any treatment? The publication Takuva S (2022) doi: 10.1097/QAI.0000000000002892 could be used to illustrate the frequency and spectrum of these reactions.

Response: Thank you for identifying this missing information. We have added details regarding the treatment and resolution of the reaction in the following sentence in page 6: "Their infusion was discontinued after receiving 60% of the intended dose; the participant was treated at the site with 2 doses of oral diphenhydramine and prescribed 2 additional daily doses of loratadine[34]; all symptoms resolved within 2 hours of the infusion." This management was in agreement with the guidelines published by Takuva, *et al*. 2022.

P9, L194-195: The text "suggesting that rectal tissue may contain some protein-rich areas that do not efficiently retain both mAbs" sounds a little mysterious to me and perhaps speculative. Could the authors either clarify or modify?

Response: We agree with the reviewer that the sentence could be more specific. However, at this point in the manuscript, we have not introduced the immunohistochemistry data, so we could not describe the protein rich areas of the tissue with low antibody (epithelium, mucosa muscularis). We have decided to remove the sentence, as the differences in antibody localization are well covered later in Figure 4 and in the discussion.

P10/11, L228-236, & Fig 5, panels B & D: Firstly, I agree that the "variable patterns … make manual scoring difficult". But in reference to Figure 5 I think the authors may be using the term 'stratum corneum' incorrectly. Ectocervical and vaginal epithelial cell layers are usually classified into 4 (or sometimes 3, or occasionally 5) layers, and referred to, in ascending order, as basal, parabasal (sometimes called spinous), intermediate (sometimes called stratum granulosum), and superficial (also known as stratum corneum). The epithelial staining in Figure 5B (both mAbs) is clearly in the intermediate (granular layer, cells with glycogen inclusions) layer, not in the stratum corneum / superficial layer. The staining in 5D (VRC01LS) is a little more complex, with strong staining in the intermediate / granular layer. There may be a little staining in the most superficial layer of the 50 um panel, but inspecting the parent 500 um panel the superficial staining looks non-specific. If the authors accept this point of correction, they will need to alter the text on P13, L292-293. I am also not convinced by the claim and illustration of differences in Figure 5 between cytoplasmic [C] vs pericellular [P] mAb localization although I do not have the benefit of in vivo high power microscopic examination.

Response: We appreciate the reviewer's concern regarding the labeling and interpretation of Figure 5, panels B and D, particularly the usage of the term stratum corneum. We agree with their definition of the layers and upon re-evaluation of the figure labeling, figure legend and main text, we noticed inconsistencies that we feel led to a misunderstanding. The labels "SC" and "B" added to panels B and D were meant solely for anatomical orientation not for the purpose of labeling where significant staining can be observed. This is inconsistent with how we used the labels "C" and "P", with accompanying arrows to indicate examples of cytoplasmic and pericellular staining. While this is explained in the figure legend, the original main text on page 10 referencing the labeling on Figure 5 was misleading. We largely agree with the reviewer's interpretation of where the predominant staining is located on these images and have made the following modifications for clarity.

1. We have removed the orientation labels "SC" and "B" and added additional labels to indicate the locations of the staining, both in the stroma and epithelium, and tied this into the main text more clearly. We have also made revisions to the figure legend on page 19 to reflect the new labeling and describe in more detail where the epithelial staining is localized (i.e., basal, parabasal, intermediate, stratum corneum). We have also replaced words in the results and discussion (page 12-13) to maintain consistent wording throughout (i.e. replaced "intracellular" with "cytoplasmic" and fixed the typo that read "paracellular".
2. We have edited the original sentence beginning on line 282 to now read "Figures 5B and 5D are higher magnification views of specific regions from Figures 5A and 5C to show examples of the localization patterns on the cellular (i.e., pericellular and/or cytoplasmic) and microanatomic level (i.e., within basal/parabasal layers, intermediate layers and/or the stratum corneum; and clustered localization). Additional examples are shown in Supplemental Figure 7."
3. We have also added a Supplemental Figure 7 to show larger, higher magnification images of 5C9 and isotype controls and additional examples to support our interpretation that "Vaginal epithelium typically displayed pronounced pericellular mAb localization, especially in the stratum corneum (Figure 5C, 5D)." The selected images also show additional clear examples of pericellular and pericellular plus cytoplasmic staining.
4.

P12, L261: The authors need to explain how they arrived at the estimate that "VRC01LS concentrations last >1 year".

Response: We have changed the sentence to "VRC01LS concentrations that last at least a year" based on the observed concentrations at Week 52 and the estimated elimination half-life.

P13, L291-2: "The greater distribution of VRC01LS suggests that FcRN aids distribution in cervico-vaginal epithelium". This statement is at odds with the authors
P12, L275-6 that mAb distribution within the cervico-vaginal epithelium is principally by convection, and not through FcRn-mediated transport, which I support. That mAb distribution within the cervico-vaginal epithelium is principally by convection, and not through FcRn-mediated transport, which I support.

Response: We agree that the statement in question does seem at odds with most of the data that supports the interpretation of that mAb distribution is principally by convection. The greater distribution of VRC01LS in the epithelium could be consistent with a role for FcRn in distribution; however, the observation could also be explained by the difference in concentration between VRC01 and VRC01LS in the serum. Thus, we have removed the sentence originally on Line 291 and replaced it with the following, starting on line 372: "FcRn may also play a role in the greater epithelial distribution of VRC01LS; however, further investigation is needed to address this possibility."

P14, L307-308: The authors state that they could not demonstrate differences between the mAbs for penetration into seminal plasma, although the half-lives are clearly different (Figure 2B, lowest panel) with p=0.067 on a small sample size, and despite their prior claims on P12, L260-261.

Response: This is a mistake on our part, and we have removed the reference to seminal plasma from that paragraph of the discussion, which was intended to focus on the cervicovaginal and intestinal comparisons between tissue and luminal secretions. Our study did not examine the urethra and associated tissue, and we do not present any evidence of FcRn-mediated recycling for this secretion.

P4, L61, & P34, Ref 21: There are doubts about the reproducibility of this work. Our own group was unable to confirm these murine findings, and the claimed phenomenon of FcRn-mediated bi-directional cervico-vaginal IgG transport clearly does not occur in humans.

Response: We appreciate the reviewer's concern about the reproducibility of this work. Indeed, we have not seen another primary manuscript showing the same bi-directional transport of cervicovaginal IgG, especially not in humans. Since the other citations already mention this study, we have removed the reference to it to avoid over-emphasis.

Reviewer #3 (Remarks to the Author):

The manuscript 'Fc-modified, HIV-1 broadly neutralizing monoclonal antibody, VRC01LS, shows enhanced biodistribution in human genital and rectal mucosa compared to VRC01 in a randomized clinical trial' by Lemos, et al presents a novel longitudinal characterization of the biodistribution and localization to mucosal sites of HIV acquisition of two clinically relevant broadly neutralizing antibodies (bNAbs) following single-dose passive immunization of men and women. The manuscript, from a top-notch group of investigators, clearly demonstrates a primary important finding -- that the

LS modification confers approximately 3-fold enhancements to bNAb Cmax and half-life in vaginal, cervical and rectal tissues, resulting in VRC01LS persisting >1 year at these relevant sites of HIV acquisition. As such, these results extend earlier reports of VRC01/VRC01LS pharmacokinetics in serum and have obvious relevance toward understanding bNAb-mediated protection at sites of HIV exposure.

In addition, the data presented raise interesting points concerning apparent underestimation of mucosal tissue-associated bNAb levels inferred from bNAb levels measured in mucosal secretions, as well as the relative paucity of bNAb within rectal glandular epithelium vs cervicovaginal eptithelium.

The authors duly note some of their studies' limitations, including the relatively small number of study participants and lack of female rectal and male genital tissues for analysis. Overall, the paper is very clearly written and the methods pleasantly contain sufficient detail.

Response: Thank you to the reviewer for the positive evaluation of our manuscript and for highlighting some of our findings and limitations.

A few suggestions that may improve the readability of the manuscript are:

1. Improve the resolution of the IHC panels presented in Figure 4A.

Response: Thanks to the reviewer for pointing this out. We believe this may have been an artifact of how the images were compressed into the PDF. We have revised Figure 4A to make it larger and with higher resolution to improve visualization.

2. Present isotype-stained control sections at high magnification, corresponding to Figure 4 panels B-E

Response: Figures 4B, 4C, 4D and 4E now include the adjacent isotype control run in parallel with the 5C9 stained section. The figure legend has been adapted as follows: "B-E) Sections from 4 different participants at high magnification, with adjacent isotype control-stained images. B), C) Sections of rectal biopsies from 2 different VRC01-infused participants at 5-6 weeks post infusion. D), E) Sections of rectal biopsies from 2 different VRC01LS-infused participants at 5-6 weeks post infusion. Markings indicate A: Adherent mucus layer, GE: glandular epithelium, LP: Lamina propria, MM: muscularis mucosa, *: selected epithelium depicting mAb staining. A 100 µm ruler indicates size."

3. To the main text, add a brief description regarding how manual scoring was performed for rectal immunohistochemistry presented in Figure 4F and a description/reference(s) to the use of H-scores regarding cervical/vaginal analyses in Figure 5E.

Response: We have added the following sentence to the results on page 10: "Manual scoring of all rectal immunohistochemistry images was conducted blinded to mAb assignment, assigning a number from 0 (lowest) to 5 (highest) according to the intensity of DAB staining (Figure 4F)". We have also added a reference for the use of H-scores after the sentence "…and H-scores (Figure 5E), which weigh the staining intensity of the positive areas." on line 295. We have also added the reference in the method section where the analysis was described (line 815).

Reviewer #1 (Remarks to the Author):
I thank the authors for taking my comments and suggestions into consideration. I feel that most aspects have been adequately addressed in the authors' response and the revised version of the manuscript.

Response: *We thank Reviewer 1 for the careful and detailed review of the manuscript. Below are the point to point responses.*

Query #1: In the added Fig. S1C, serum concentrations of VRC01 and VRC01-LS are now presented in "µg/mL". In Fig. 2A, they are displayed as "µg/ml per kg".

Comparing the values in the individual graphs, something seems to be off. For example, VRC01-LS concentrations in males at week 13-14 appear to be essentially identical (ca. 1) in both the "µg/ml per kg" (Fig. 2A) and "µg/ml" units (Fig. S1C) – which I believe cannot be correct. I suggest double-checking values, curves, and axis labels.

Moreover, the absolute values written out ("median cmax") should match the graph units. For example, in Fig. 2A, the median cmax (determined at wk 1-2) in males are given as 240.5 and 731.1 for VRC01 and VRC01-LS, respectively. However, these values are way above what is displayed in the graphs (cmaxes <10). In Fig. S1C, the median cmaxes in males are given as 0.241 and 727 for VRC01 and VRC01-LS, which would be a >1.000-fold difference. I suggest double-checking all numbers.

Response #1: *We appreciate your calling our attention to this mistake. In Figure 2, the values reported as Cmax had not been weight normalized and these values are now corrected. We have also corrected Figure S1 and the values now match the figures.*

Query #2: The AE table (Table S3) has been updated but I feel it can have a bit more clarity.

The separation between related and unrelated AEs could be clearer. Below the heading "Participants with one or more AEs" only two participants are listed at first (presumably those with related AEs). Perhaps it would be sufficient to change the headings to "Participants with Study Drug-Related AEs", "Type and Severity of Study-Drug Related AEs", and "Participants with AEs not Considered Study-Drug Related"?

Moreover, I would suggest to rephrase the line "Number (n)" to "Participants (n)". Currently it suggests that these numbers indicate the number of AEs as they are listed right under the heading "Study related and Unrelated Adverse Events".

Response #2*: We have revised the Table S3 for clarity. Study related AEs are listed first including symptoms and grading. Study unrelated AEs are listed second. Total participants has been removed as it has been described in Figure 1, and the numbers in the table are described in the heading as "Number of participants with AEs (% of total)".*

Query #3: In Figure S5 (previously Figure S3), the y-axis has been updated to indicate that only VRC01 levels are shown. If no VRC01-LS levels are included, then this should also be removed in the legend. In the sentence “Correlations [...] in male participants IV infused with VRC01 or VRC01LS [...]” that starts the legend, the “or VRC01LS” should be removed.

Response #3: *As recommended, VRC01LS has been removed from the Figure S5 legend.*

Query #4: Remarks on code availability:

I believe there is no code available (and I was not expecting any code).

Response #4: *We had uploaded all relevant code to FigShare via the journal, so the code should be available.*

Reviewer #3

Thank you for your manuscript revisions - all of my suggested changes have been incorporated.

Editorial note: this reviewer was additional asked to comment in place of reviewer 2 who was unavailable to provide comment at this stage. And stated "I feel that Reviewer #2’s concerns have been fully addressed in the revised manuscript."

Response: *We thank Reviewer 3 for their effort to review the document and address Reviewer 2 and 3 considerations.*