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Peer Review File

NK Cell Depletion in Bispecific Antibody Therapy is Associated with Lack of HIV Control after ART interruption

Corresponding Author: Dr Maria J Buzon

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript submitted by Buzón and collaborators describes in vitro and in vivo evaluation of a tetravalent bispecific antibody (Bi-Ab32/16) that is designed to engage the HIV envelope protein at the gp120 A32 epitope, and CD16 on NK cells. The authors demonstrate that Bi-Ab32/16 has potent in vitro ADCC activity against cells coated with HIV envelope, infected with HIV virus, and against reactivated patient-derived latent virus-infected cells. Much of this work represents an incremental advancement beyond that previously demonstrated for the A32 antibody itself or for other A32-CD16 bispecifics in published studies. However, the work is comprehensively done and includes some unique and relevant findings such as the ability of the bispecific, but not the parental IgG antibody, to eliminate cells coated with HIV-1 envelope when interacting with NK cells from PWH on ART. Based on the strong in vitro findings, the ability of Bi-Ab32/16 to delay viral reactivation or control VL after ATI was modeled in the ART-treated Hu-NSG-Tg (IL-15) mouse model. Unexpectedly, this model indicated modest delay in virus recrudescence and some evidence of virus control in the group of mice treated with the A32 IgG antibody (a potentially novel finding as this may have never have been tested/demonstrated for an antibody without neutralization ability?), but no benefit for those treated with the more potent Bi-Ab32/16. This was suggested to be a result of reduced NK cells in the Bi-Ab32/16 group, which may be a result of overactivation due to synergy from Bi-Ab32/16 NK activation in the presence of NK activation due to virus reactivation. Overall, the manuscript is clear and helps fill a gap in knowledge of whether CD16 bispecifics may be advantageous in the setting of ART interruption. There are several issues that should be resolved to improve the manuscript:

- Why is A32 IgG not included as a comparator in Figure 1 F-I? Comparisons between the antibody and bispecific are otherwise made throughout the manuscript.
-what is the source of the HIV-1BAL? Is this an engineered infectious clone or a more ‘wild-type’ virus?
-how is normalization done in Figure 2H?
-Figure 6, why is there missing data from one Bi-Ab32/16 mouse and one A32 mouse?
-Figure 6D, most of these correlations are non-significant, and most of the r values are below 0.5, conclusions based on these findings should be tempered accordingly.
-Fix legend in Figure2A.
- Per PMID: 36111287, an A32-based bispecific has initiated human clinical testing. It would be helpful to include this in the discussion.
-A concern with the A32 epitope is that although it may allow early recognition of infected cells, the window of opportunity may be very limited due to CD4 shedding. Might the in vivo model be more sensitive to this compared to the crowded in vitro infection where there may be bystander cell CD4 and envelope interactions?
-The potential for the Bi-Ab32/16 to work in combination with other antibodies should be discussed. This could help overcome the problem of a narrow window of opportunity, and relevant literature supporting A32 improving activity of combinations should be discussed (see PMID: 32584790).
-an A32 bispecific has been previously demonstrated to eliminate reactivated patient-derived latent cells, consider citing PMID: 26413868
-The increase in CD16 in Supplementary Figure 3 is surprising, normally activation of NK cells through CD16 causes ADAM17 dependent CD16 cleavage. Was this observed with the A32 IgG? Was the MFI of CD16 on a per cell basis changed? What is the proposed mechanism of CD16 increase here?
-Figure 4 is an important aspect of the paper as this is the only data demonstrating that the Bi-Ab32/16 is able to direct NK cells to kill cells infected with patient derived virus. It would not be expected for there to be many p24+ CD4 T cells after only 18 hours of activation. What were frequencies of p24+ in these individuals? That is, how robust is this data, are results based on loss of only a few p24+ cells?
-Has an antibody lacking neutralization been shown to delay virus reactivation in any preclinical model? If not, are the findings from this study for A32 significant when compared to control animals (22 days vs 15, control in 2/6)? If so, this

should be given some discussion even without it having been a primary goal of the study it seems it may be an important observation.

Reviewer #2

(Remarks to the Author)
The Authors report some interesting findings on the potency of a new bi-specific mAb that recognizes an immunodominant epitope of the HIV-1 envelope in the CD4-inducible constant region 1 and 2. The new molecule is compared to the parental A32 mAb. They observed that the new molecule is more potent than the parent molecule when tested against gp120-coated target cells as well as against some of the infected target cells, some of which were also generated ex-vivo from samples obtained from people living with HIV. These findings are not recapitulated by the in vivo studies in a humanized mouse model. In addition, the Authors report that after infusion in the infected mice, they observed a declined in the level of NK and a poor control of virus rebound upon ART interruption. The NK decline is suggested to be the cause for the lack of control.
A few points should be addressed by the Authors.
1) In the heat map provided in Figure 1, it is not clear how little differences are observed between the cells in the control conditions as supposed to those exposed to the bispecific mAb. Moreover, it does not seem that the CD16 receptor is down regulated. Can the authors provide some comments about these aspects of their findings?
2) Have the Authors determined if the bispecific mAb can bind the cells of the infected cells in the humanized mice like what done with samples in Figure 3?
3) The Authors indicate that there is a significant negative association between circulating CD56+ cells and HIV RNA in Figure 5D. However, the r=-0.52 is not a strong correlation. The -0.52 values indicates more of a trend than an actual negative correlation.
4) There is no discussion between the observation with the binding to infected cells and their functional cytotoxic results and what observed by Finzi et al who repeatedly report how CD4-downregulated cells are not susceptible to the activity of the A32 class of mAb.
5) The Authors should should also include a discussion of their findings in view of what reported by Koup and collaborators that NK cells also declined in the circulating PBMC upon infusion of CD4bs VRC01 mAb with an optimized Fc region.
In general, the authors should review the grammar and spelling in the manuscript as there are a few typos either in the main text or in the legends.

Version 1:

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Reviewer #1

(Remarks to the Author)
The revised manuscript addresses prior concerns. One additional modification would be to include the representative flow plots for Figure 4 that were submitted with the rebuttal letter into that figure in the final manuscript, or at minimum, as a supplemental figure.

Reviewer #2

(Remarks to the Author)
The authors have done quite a nice job in addressing the reviewers' comments. The manuscript is still worth to be considered for publication. That said it seems ver sad that they still don't understand that a r=-0.52 is NOT meaningful. They should have an appropriate discussion with a statistician who can explain what a meaningful correlation exactly means and that it is not related to a p value, which can be significant even with r=0.1.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript submitted by Buzón and collaborators describes in vitro and in vivo evaluation of a tetravalent bispecific antibody (Bi-Ab32/16) that is designed to engage the HIV envelope protein at the gp120 A32 epitope, and CD16 on NK cells. The authors demonstrate that Bi-Ab32/16 has potent in vitro ADCC activity against cells coated with HIV envelope, infected with HIV virus, and against reactivated patient-derived latent virus-infected cells. Much of this work represents an incremental advancement beyond that previously demonstrated for the A32 antibody itself or for other A32-CD16 bispecifics in published studies. However, the work is comprehensively done and includes some unique and relevant findings such as the ability of the bispecific, but not the parental IgG antibody, to eliminate cells coated with HIV-1 envelope when interacting with NK cells from PWH on ART. Based on the strong in vitro findings, the ability of Bi-Ab32/16 to delay viral reactivation or control VL after ATI was modeled in the ART-treated Hu-NSG-Tg (IL-15) mouse model. Unexpectedly, this model indicated modest delay in virus recrudescence and some evidence of virus control in the group of mice treated with the A32 IgG antibody (a potentially novel finding as this may have never have been tested/demonstrated for an antibody without neutralization ability?), but no benefit for those treated with the more potent Bi-Ab32/16. This was suggested to be a result of reduced NK cells in the Bi-Ab32/16 group, which may be a result of overactivation due to synergy from Bi-Ab32/16 NK activation in the presence of NK activation due to virus reactivation. Overall, the manuscript is clear and helps fill a gap in knowledge of whether CD16 bispecifics may be advantageous in the setting of ART interruption. There are several issues that should be resolved to improve the manuscript:

- Why is A32 IgG not included as a comparator in Figure 1 F-I? Comparisons between the antibody and bispecific are otherwise made throughout the manuscript.

We thank the reviewer for this suggestion. The experiments in Figure 1F-I focused on characterizing the phenotypic changes in NK cells induced by the bispecific antibody. A32 IgG was not included as a comparator because analyzing its effect on NK cell phenotype was not the main objective of this study. Our research builds on prior work from our lab, where A32-containing nanoparticles were shown to promote doublet formation (PMID: 34394703), and existing literature, which has extensively described the ADCC response and NK cell activation induced by A32 IgG (PMID: 21543485). Given this context, we prioritized exploring the novel effects of the Bi-Ab32/16, while comparisons with A32 IgG

were included elsewhere in the manuscript to evaluate functional outcomes, such as killing efficiency of HIV-infected cells.

-what is the source of the HIV-1BAL? Is this an engineered infectious clone or a more 'wild-type' virus?

Thank you for your comment. The HIV-1 BaL strain is a laboratory-adapted virus derived from subtype B HIV and is characterized by its exclusive use of the CCR5 co-receptor for cell entry, classifying it as an R5-tropic virus (Propovic et al., 1988). It was originally isolated from infant lung tissue, serving as its source. While it is not inherently more infectious than wild-type HIV strains, HIV-1 BaL is widely used in research due to its consistent R5-tropism, which makes it particularly valuable in studies focusing on the early stages of HIV infection and transmission, where R5-tropic viruses are predominantly involved.

-how is normalization done in Figure 2H?

The data in Figure 2H is normalized to the basal killing condition, which represents the natural NK cell response in the absence of ADCC-mediating antibodies. Normalization was performed by subtracting the percentage of killing observed in the basal condition from the percentage of killing obtained in each experimental condition. This approach allows for the calculation of the net ADCC response, isolating the effects of HIV plasma or ADCC-mediating antibodies on NK cell activity. We have included this explanation in the Figure 2 legend.

-Figure 6, why is there missing data from one Bi-Ab32/16 mouse and one A32 mouse?

Thank you for pointing this out. Two samples were excluded from the analysis due to a low number of cells obtained after the experimental procedure, which did not meet the criteria for reliable data analysis. We have revised the Methods section and included the following explanation for clarity and transparency: *"Of note, two spleen samples from 11 WPI, one from the A32-treated group and one from the Bi-Ab32/16-treated group, were excluded from the final analysis due to an insufficient number of cells obtained after the experimental procedure (less than 50 cells). This criterion was applied to ensure the reliability and consistency of the data".*

-Figure 6D, most of these correlations are non-significant, and most of the r values are below 0.5, conclusions based on these findings should be tempered accordingly.

We thank the reviewer for the suggestion. The lack of statistical significance in most of the correlations in Figure 6D is expected given the low number of mice included in the experiment. We agree that the conclusions drawn from these findings should be tempered accordingly. To address this, we have revised the Results section to add clarity. We acknowledge this limitation in the manuscript and state the following: "*Notably, some of the associations presented in Figure 6D did not achieve statistical significance, likely due to sample size limitations; therefore, while the results suggest a potential relationship, definitive conclusions cannot be drawn from the current dataset".*

-Fix legend in Figure2A.

We apologize for this inconvenience. The legend on Figure 2A has been modified appropriately.

- Per PMID: 36111287, an A32-based bispecific has initiated human clinical testing. It would be helpful to include this in the discussion.

We appreciate this suggestion. As recommended, we have included the results of the first-in-human Phase 1 safety study of the MGD014 antibody in the discussion: *"Moreover, our findings align with the ongoing development of A32-based bispecific antibodies (A32-CD3) for HIV cure strategies, exemplified by MGD014, which has progressed to human clinical testing (NCT03570918). The completed first-in-human Phase 1 safety study demonstrated the safety and tolerability of MGD014 in PLWH on ART (PMID:* 36111287*), supporting the potential of A32-based bispecific antibodies potential for further clinical development to target persistent HIV infection".*

-A concern with the A32 epitope is that although it may allow early recognition of infected cells, the window of opportunity may be very limited due to CD4 shedding. Might the in vivo model be more sensitive to this compared to the crowded in vitro infection where there may be bystander cell CD4 and envelope interactions?

Thank you for your insightful comment. Despite a limited window of opportunity, strategies targeting this epitope have been explored due to its importance in virus entry and during virus budding (PMID: 31338095, 11602749). Notably, the A32 epitope has been successfully targeted in the past (PMID: 36111287). However, studies have reported that HIV restricts Env-CD4 interaction by reducing CD4 expression and limiting Env accumulation on the surface of HIV infected cells, therefore underscoring a potential limitation of the A32 epitope. We agree with the reviewer that this phenomenon could be more pronounced in the in vivo model, where the dynamic microenvironment might amplify the sensitivity to CD4 loss. In contrast, in vitro infection systems, characterized by

higher cell densities, may allow bystander cell interactions between CD4 and the viral envelope, potentially mitigating the effects of CD4 shedding. This difference underscores the need to consider the distinct contexts of in vivo and in vitro systems when interpreting the efficacy of A32-targeted interventions. As recommended by the reviewer, we have included the following sentence to the Discussion section: *"We hypothesize that this limitation could be more pronounced in vivo, where the dynamic microenvironment and reduced bystander cell interactions may heighten sensitivity to CD4 loss, compared to in vitro systems where higher cell densities allow more frequent interactions between CD4 and the viral envelope".*

-The potential for the Bi-Ab32/16 to work in combination with other antibodies should be discussed. This could help overcome the problem of a narrow window of opportunity, and relevant literature supporting A32 improving activity of combinations should be discussed (see PMID: 32584790).

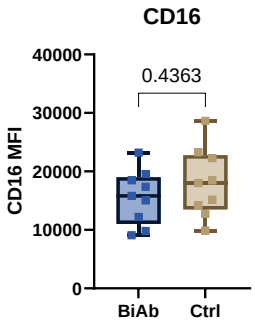
We appreciate the reviewer's valuable comment. Previous in vitro studies have demonstrated that combinations of three monoclonal antibodies (mAbs) can mediate over 30% of NK cell-mediated killing of HIV-infected cells (PMID: 32584790). We concur with the reviewer that the enhanced ADCC responses observed with the bispecific antibody, along with the delayed viral rebound achieved by the A32 parental antibody alone, strongly support the rationale for combining distinct non-neutralizing antibodies as a strategy for HIV treatment. To reflect this idea, we have revised the discussion section as follows: *"In addition, strategies to address the challenge of a narrow window of opportunity could include the combination of distinct non-neutralizing antibodies, as suggested by previous studies (PMID: 32584790). Administering appropriate combinations of mAbs with diverse specificities could maximize the recognition and effective elimination of infected cells"*

-an A32 bispecific has been previously demonstrated to eliminate reactivated patient-derived latent cells, consider citing PMID: 26413868

We appreciate the reviewer's suggestion. As recommended by the reviewer, we have included the following sentence in the discussion section: *"Our findings are consistent with previous studies highlighting the potential of A32-based bispecific molecules to target and eliminate latently infected cells from PWH upon latency reversal (PMID: 26413868)."*

-The increase in CD16 in Supplementary Figure 3 is surprising, normally activation of NK cells through CD16 causes ADAM17 dependent CD16 cleavage. Was this observed with the A32 IgG? Was the MFI of CD16 on a per cell basis changed? What is the proposed mechanism of CD16 increase here?

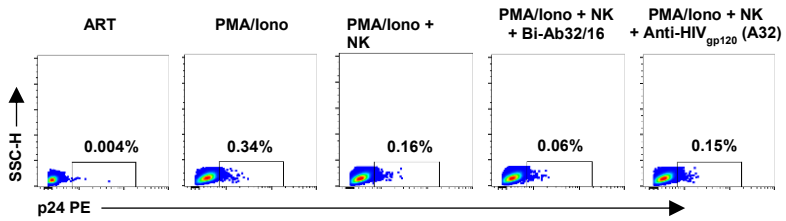
We thank the reviewer for their insightful comment. Regarding the role of A32 IgG, we did not include this condition in this specific experiment, as the primary objective was to assess whether the Bi-Ab alone could modulate the NK cell phenotype. Previous studies have already described the effects of A32 IgG on NK cell activation and ADCC, providing a robust foundation for comparison in separate functional assays. Regarding the MFI of CD16, we have thoroughly reviewed our data, and we did not observe any statistical changes among the conditions (see below).



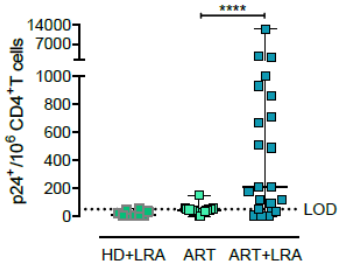
It is indeed well-documented that activation of NK cells through CD16 can lead to its downregulation via ADAM17-dependent cleavage, often observed as early as 5 hours after interaction with certain target cells. However, in our experimental setup, isolated NK cells were stimulated with the bispecific antibody in the absence of target or additional cells. This suggests that CD16 recognition could occur without the typical activation and cleavage processes associated with target cell engagement. We hypothesize that the observed increase in CD16 expression might indicate that NK cells are being primed for enhanced functionality, particularly in their ADCC capability. Elevated CD16 levels could improve the capacity of NK cells to mediate ADCC in subsequent encounters with target cells. Furthermore, the concurrent upregulation of NKG2C and CD57 suggests the possibility of a memory-like phenotype being induced in these NK cells, which aligns with reports of adaptive NK cell responses upon target restimulation.

-Figure 4 is an important aspect of the paper as this is the only data demonstrating that the Bi-Ab32/16 is able to direct NK cells to kill cells infected with patient derived virus. It would not be expected for there to be many p24+ CD4 T cells after only 18 hours of activation. What were frequencies of p24+ in these individuals? That is, how robust is this data, are results based on loss of only a few p24+ cells?

We thank the reviewer for their observation and agree that the frequencies of p24+ cells following pharmacological reactivation of the latent reservoir for 18h are indeed minimal, ranging here from 0.05% to 0.68%. A representative flow cytometry plot is presented below for reference:



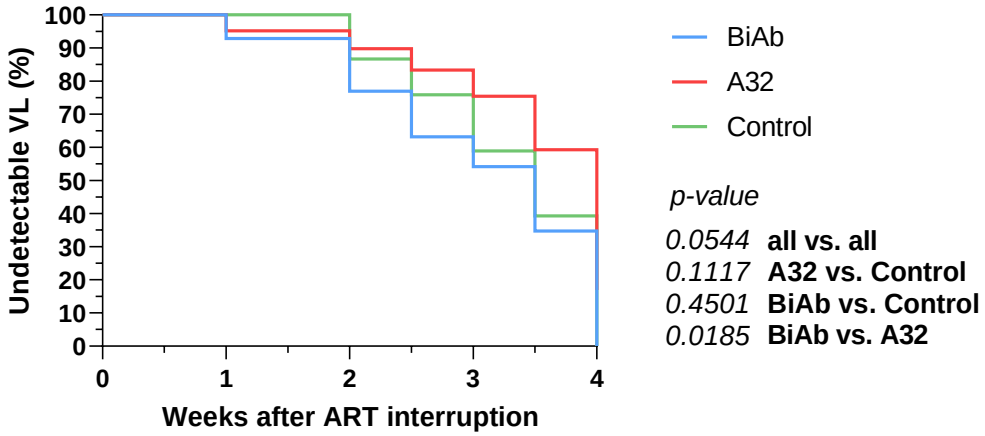
As described in our previous work (PMID: 35616530, Fig 2B), the limit of detection for the assay was established at 50 $p24^+$ cells per million $CD4^+$ T cells (Figure below). This threshold reflects the sensitivity of the method and underscores the challenges inherent in detecting rare events such as reactivated cells from the latent HIV reservoir.



-Has an antibody lacking neutralization been shown to delay virus reactivation in any preclinical model? If not, are the findings from this study for A32 significant when compared to control animals (22 days vs 15, control in 2/6)? If so, this should be given some discussion even without it having been a primary goal of the study it seems it may be an important observation.

To our knowledge, there is no clear evidence to date demonstrating that an antibody lacking neutralization ability can delay HIV reactivation in preclinical models.
In our study, while the A32-treated group exhibited a delayed time to viral rebound compared to the control group (22 days vs. 15 days; viral rebound in 4/6 mice compared to 5/5 in the control condition), the difference did not reach statistical significance (p=0.11) (Figure below). We acknowledge that the imbalance in group size, with one fewer subject in the control arm, might have affected the statistical power to detect significance. Nonetheless, the observation that the A32-treated group experienced a longer time to viral rebound compared to the bispecific antibody-treated group – contrary to the trends observed in vitro – represents an intriguing and potentially important finding that suggest an effect of non-neutralizing antibodies controlling viral rebound in conjunction with NK cells (present in the NSG-IL15 model), more likely via ADCC. These results are in line with Nussenzweig results´s (PMID: 28757252) that showed an effect of an anti-HA antibody (non-neutralizing) in HIV infection (using an engineered HIV clone expressing an HA epitope on the surface). In addition, the capacity of A32 to delay viral rebound agrees with the fact that anti-V1V2 non-neutralizing IgGs were associated with protection in the RV144 clinical trial (PMID: 22475592).

As suggested by the reviewer, we have revised the discussion of these results to highlight the complexity of the in vivo context and the interplay of antibody function beyond neutralization in delaying viral reactivation: *"Furthermore, in our study, the A32-treated group exhibited a delayed viral rebound compared to the Bi-Ab32/16-treated group, contrasting with the findings observed in vitro and highlighting an intriguing and potentially significant observation. The extended rebound time suggests that A32 may induce a more physiological response, potentially avoiding the adverse effects linked to the robust stimulation caused by the Bi-Ab32/16. These findings underscore the complexity of translating in vitro results to in vivo contexts, where immune dynamics and tissue distribution significantly influence antibody function. Our results are in line with other studies that reported the in vivo antiviral effect of non-neutralizing antibodies (PMID: 28757252) and with the fact that non-neutralizing IgGs targeting V1V2 epitopes were associated with protection in the RV144 clinical trial (PMID: 22475592)."*



Reviewer #2 (Remarks to the Author):

The Authors report some interesting findings on the potency of a new bi-specific mAb that recognizes an immunodominant epitope of the HIV-1 envelope in the CD4-inducible constant region 1 and 2. The new molecule is compared to the parental A32 mAb. They observed that the new molecule is more potent than the parent molecule when tested against gp120-coated target cells as well as against some of the infected target cells, some of which were also generated ex-vivo from samples obtained from people living with HIV. These findings are not recapitulated by the in vivo studies in a humanized mouse model. In addition, the Authors report that after infusion in the infected mice, they observed a declined in the level of NK and a poor control of virus rebound upon ART interruption. The NK decline is suggested to be the cause for the lack of control. A few points should be addressed by the Authors.

1) In the heat map provided in Figure 1, it is not clear how little differences are observed between the cells in the control conditions as supposed to those exposed to the bispecific mAb. Moreover, it does not seem that the CD16 receptor is down regulated. Can the authors provide some comments about these aspects of their findings?

We appreciate the reviewer's observation. The heatmap in Figure 1H illustrates the global differences in the mean expression of each marker across conditions and clusters, as automatically generated by the OMIQ software. This heatmap assigns the average receptor expression per cluster and condition, providing an overview of the NK cell phenotypic profile. Markers with distinctly high or low expression contribute most significantly to cluster identification, while those with more variable expression are assigned subsequently. To complement this analysis, Figure 1I specifically depicts the frequency of each cluster across conditions, and Supplementary Figure 3 shows the frequency of each marker in total NK cells. The latter figure is particularly useful for discerning subtle differences in marker expression between the control and bispecific antibody-exposed conditions at the level of individual study samples. Regarding CD16 expression, Figure 1H shows no visible differences between conditions across the different clusters; however, it should be considered that the frequency of each cluster varies between conditions, as shown in Figure 1I. This variation could explain the overall higher CD16 expression observed in total NK cells stimulated with the bispecific antibody at 24 hours compared to the control condition, as shown in Supplementary Figure 3.

2) Have the Authors determined if the bispecific mAb can bind the cells of the infected cells in the humanized mice like what done with samples in Figure 3?

We thank the reviewer for their suggestion. Unfortunately, we were unable to perform the same experiments to assess whether the bispecific antibody bound to infected cells in the humanized mice, as was done in the in vitro assays in Figure 3, due to the limited quantity of sample available. Specifically, the volume of blood obtained from these small animals was insufficient to conduct additional binding assays alongside the required flow cytometric analyses. This inherent limitation of working with mice restricted our ability to fully address this aspect in the current study. We agree with the reviewer that performing such experiments would have been valuable to complement our findings and provide further insights into the binding properties of the bispecific antibody in vivo.

3) The Authors indicate that there is a significant negative association between circulating CD56+ cells and HIV RNA in Figure 5D. However, the r=-0.52 is not a strong correlation. The -0.52 values indicates more of a trend than an actual negative correlation.

We thank the reviewer for their valuable comment. We agree that the r-value of -0.52 in our results represents a trend rather than a strong negative correlation. However, we believe that working with animals introduces complexities that make achieving strong correlations challenging. Taking into account that the p-value is significant, we consider the results to be meaningful.

4) There is no discussion between the observation with the binding to infected cells and their functional cytotoxic results and what observed by Finzi et al who repeatedly report how CD4-downregulated cells are not susceptible to the activity of the A32 class of mAb.

We thank the reviewer for their suggestion. Andrés Finzi and colleagues have repeatedly demonstrated that HIV-mediated CD4 downregulation serves as an immune evasion mechanism, protecting infected cells from ADCC mediated by non-neutralizing antibodies like A32. This mechanism is particularly critical because CD4 downregulation induces a conformational shift in the HIV Env, adopting a closed conformation that escapes recognition by A32 and other non-neutralizing antibodies. Consequently, productively infected cells become resistant to ADCC.
In our study, we were concerned of this limitation, which is why we tested the binding capacity of our bispecific antibody, containing the A32 antibody, and the parental antibody A32 alone, in productively infected cells. As detailed in the manuscript, we performed ex vivo infection of isolated CD4+ T cells with a CCR5-tropic HIV strain and cultured them for five days. Under these conditions, we observed that the majority of p24+ cells retained CD4+ expression, and nearly 80% of these cells effectively bound both Bi-Ab32/16 and A32 antibodies, confirming that our approach targeted early stages of HIV infection before significant CD4 downregulation occurred. Furthermore, previous studies from our group (PMID: 28698276) confirmed that productively infected cells maintain CD4 expression under similar conditions. Importantly, we do not activate the cells with PMA, PHA or CD3/CD28 antibodies before infection. In contrast, studies by Finzi et al., utilized the HIV NL4.3 strain, known for higher infectivity, and employed an 8-day infection culture period with cells previously activated, allowing sufficient time for extensive CD4 downregulation. These differences in viral strain and experimental protocols likely explain the discrepancies observed between our findings and those reported by Finzi et al.
We agree with the reviewer that this is a critical point to address and have revised the discussion to include the following statement: "*Notably, while previous studies have identified HIV-mediated CD4 downregulation as an immune evasion mechanism limiting the efficacy of A32-based antibodies (PMID: 30234611, 36640355), our experimental data confirms that our approach effectively targets the early stages of HIV infection, prior to significant CD4 downregulation. Variability in susceptibility to A32-mediated ADCC across studies can likely be attributed to differences in viral strains and infection protocols, including culture duration, strain-specific dynamics of CD4 downregulation, and prior cell*

activation. In our system, the partial preservation of CD4 expression on productively infected cells underscores the potential efficacy of A32-based bispecific antibodies, particularly in addressing early phases of HIV infection".

5) The Authors should should also include a discussion of their findings in view of what reported by Koup and collaborators that NK cells also declined in the circulating PBMC upon infusion of CD4bs VRC01 mAb with an optimized Fc region.

We thank the reviewer for the comment. We have extensively revised the literature reported by Koup and we have not found the mentioned study. In one study, they observed that non-human primates (NHP) treated with the VRC07 antibody – an optimized Fc variant of VRC01 – exhibited a more rapid viral rebound compared to those treated with VRC01 (PMID 39198422), however no depletion of NK cells was observed. Our study, however, aligns with observations that bispecific antibodies targeting CD16 can result in a reduction of NK cell frequencies, a phenomenon attributable to a fratricide mechanism (PMID 31172847). We have revised the discussion section to incorporate the following statements: *"Of note, an in vitro study demonstrated that multivalent, high-affinity binding of antibodies to CD16 poses a risk of cross-linking the receptor across multiple NK cells (PMID: 31172847). This cross-linking can trigger CD16a-mediated cytotoxicity, resulting in the depletion of the NK cell population. While the study noted that fratricide is less likely with antibodies containing only a single binding site for CD16a, it also highlighted that the proportion and extent of NK cell fratricide could be influenced by the antibody format and the variable domain order of the anti-CD16a antibody. Specifically, the study reported that Fab-based CD16a bispecific antibodies were found to be more prone to NK cell depletion. These findings and ours underscore the critical need to carefully evaluate the long-term in vivo effects of bispecific antibodies like Bi-Ab32/16, particularly their impact on NK cell dynamics, to ensure that therapeutic strategies leveraging CD16-mediated mechanisms do not inadvertently compromise immune control of HIV infection."*

In general, the authors should review the grammar and spelling in the manuscript as there are a few typos either in the main text or in the legends.
Done

Reviewer's comments:

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The revised manuscript addresses prior concerns. One additional modification would be to include the representative flow plots for Figure 4 that were submitted with the rebuttal letter into that figure in the final manuscript, or at minimum, as a supplemental figure.

As suggested by the reviewer the representative flow plots have been included in the final version of the manuscript as a Supplementary Figure 6.

Reviewer #2 (Remarks to the Author):

The authors have done quite a nice job in addressing the reviewers' comments. The manuscript is still worth to be considered for publication. That said it seems ver sad that they still don't understand that a r=-0.52 is NOT meaningful. They should have an appropriate discussion with a statistician who can explain what a meaningful correlation exactly means and that it is not related to a p value, which can be significant even with r=0.1.

We thank the reviewer for their valuable comment. To address this, we have revised the results section of the manuscript to provide greater clarity and have included the following statement: "Additionally, CD56 + NK cell frequencies displayed a trend of negative correlation with HIV infection levels at the end of the experiment".