EMBO reports

Anti-HIV-1 antibodies trigger non-lytic complement deposition on infected cells

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Transaction Report: This manuscript was transferred to *EMBO reports* following peer review at *The EMBO Journal.*

1st Editorial Decision 26 September 2019

Thank you for the transfer of your research manuscript to EMBO reports. I now went through your paper, the referee reports from The EMBO Journal (attached below), and your 'preliminary point-by-point response'. All referees acknowledge the potential interest of the findings. Nevertheless, the three referees have also raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here. Looking at the reports, I feel that a significantly revised manuscript could be suitable for publication at EMBO reports, provided the major referee concerns are addressed.

As EMBO reports emphasizes novel functional over detailed mechanistic insight, we will not require to address points regarding more refined mechanistic details. All other concerns, and all technical points need to be addressed, though, as we require strong in vivo relevance of the findings, and clear experimental support of the major conclusions. Having said this, the revision as suggested by your preliminary p-b-p-response seems very reasonable.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be fully addressed in the revised manuscript (as detailed above) and in a complete point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review (using the same referees that have assessed the study at The EMBO Journal). It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient

for the revisions so that we can discuss the revisions further.

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When submitting your revised manuscript, please also carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision. When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that the changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

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The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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Moreover, I have these editorial requests:

5) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

6) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data

can be accessed at the end of the reference. Further instructions are available at:
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7) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

8) Please format the references according to our journal style. See:
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9) Please add up to 5 key words to the title page.

10) Please move the Material & Methods section up and place it after the Discussion.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Dufloo et al. describe a series of experiments in which they measure the deposition of C3 on cells expressing HIV ENV (either ectopically or because of viral infection) after incubation with anti-ENV antibodies. Despite C3 and C5b-9 deposition being enhanced by one of the antibodies, no CDC killing was observed. The reasons for this are not clear. However, there is evidence that C3 deposition may accelerate the disappearance of infected cells through an unknown mechanism. The possible control of HIV infection by complement is an important area of study, in particular when induced by bNAbs. This is because the production of bNAbs through vaccination remains a major international goal. The data presented by the authors are therefore important to consider and are of broad interest to the field.

The following could be addressed in a revised manuscript and would help solidify the results:

1. Figure 1B Raji B cells express Fcgamma receptors, which will bind to antibodies via their Fc. Repeating the experiment in cells that aren't expressing ENV would be a useful control.

2. Figure 1D A very high concentration of anti-C3 antibody was used in this experiment (only 1:50 dilution), which makes the possibility of cross-reaction with antibodies on the surface of the Raji cells a concern. To verify these results, the experiment should be repeated using heat inactivated serum.

3. Figure 3 T cells also can express FcgRs; I'm therefore surprised that there was no binding measured with the control antibody mGO53. Is the control antibody properly folded and functional? Is it capable of binding antibody receptors?

4. Figure 3 D&F: It would be helpful to show which specific antibodies did not give statistically significant data and/or mention this in the text. For example, it looks like only 10-1074 gave significant C5b-9 deposition.

5. Supplementary Figure 3B: anti-CD52 triggers ADCC in addition to CDC, so use of this control may not indicate that it is complement killing the cells in this experiment. This should be addressed by repeating with heat-inactivated serum. It's important that these positive controls can be relied upon to demonstrate that CDC of the cells is possible.

6. Figure 4. The data indicate that Vpu limits complement deposition but this may simply be

because Vpu limits the amount of Env expressed at the cell surface. The amount of Env expressed on the cell surface after infection with wild-type veruses deltaVPR virus should be compared and correlated with complement deposition.

7. Figure 5: I don't think the FACS plots are really necessary. The data should instead be graphed (as in 5C) and statistics applied to each day. This will allow significance to be more easily assessed.

8. Figure 7: The mechanism by which Vpu and Nef limit C3 deposition should explored. Is this simply by limiting ENV expression? Again the expression of ENV should be compared under the different conditions and correlated with C3 deposition.

The findings of this study are slightly because no CDC of infected cells was demonstrated for anti-ENV antibodies. Thus it is difficult to know how important Vpu or Nef antagonism is in limiting C3 deposition. Possibly this could be partially addressed by repeating the experiments in Figure 5 using viruses where Vpu or Nef has been deleted and determine whether this alters persistence of infected cells in the presence of the anti-ENV antibodies.

Referee #2:

Dufloo et al describes how a number of broadly neutralizing HIV-1 antibodies activates the complement system on cells engineered to express high levels of ENV and on primary CD4+ cells infected with HIV-1. Understanding how antibody effector functions, including complement, contributes to protection against HIV-1 is important to inform on future therapeutic strategies and vaccine design. The authors show that only a subset of bNAbs activate complement and that complement is not potently activated due to low level of ENV expression which are regulated by Nef and Vpu. Furthermore, they show that expression of CD59 on CD4+ T-cells counteract CDC. The conclusions regarding complement activation are supported a large number of solid experiments and agree with the current concepts of antibody mediated complement activation. The text and figures are well composed and easy to follow even for the non-expert. Whereas the presented work could be of significant interest for an HIV oriented audience it is possibly too specialized for a broader audience including complement focused readers.

Minor issues

1. It would be helpful if background on Nef and Vpu regulation of ENV expression was presented in the introduction or at first mention under results as this is important for the broader audience.
2. It could strengthen the manuscript if the discussion considered whether combinations of bNAbs could increase CDC (as observed for antibodies against EGFR). Is the amount of ENV on infected CD4+ cells simply too low to effectively to activate complement no matter what antibody that is used?
3. The current manuscript very much appeals to an HIV oriented audience, whereas the complement perspective is less well covered.
4. Abstract "Here, we show that a subset of broadly neutralizing antibodies (bNAbs), targeting the CD4 binding site and the V3 loop". Already here the non-HIV reader is lost, what is the antigen?
5. A better presentation of C3, C3b regulation on cells and how C3b density correlates with C5 convertase and terminal pathway initiation is needed. In fact, what is measured during "C3 deposition" are its degradation products C3b, iC3b.
6. On p8, the "open conformation of Env" is mentioned, what is this and how does it differ from other Env conformations?
7. Discussion "Recognition of complement components by CR2 on B cells decreases the threshold of BCR stimulation, favoring an optimal response". CR2 only recognize iC3b and C3dg.
8. Methods: Provide the specificity of the C3 antibody used here for detection
9. Fig 1 legend "the MFI the of no antibody" needs correction
10. Fig S2. The legends says red curve, it is not red.

Major issues

1) Given the current understanding of antibody mediated complement activation, the lack of complement activation on cells expressing low amount of antigen (ENV) is to be expected. The obtained results thus support previous observations but also lack novelty. The work presented is very descriptive, but lacks mechanistic models at the molecular level that are evaluated by experiments. The most important example of this is the lack of attempt to explain why sublytic MAC formation induce disappearance of infected T-cells. The experimental work is very much based on FACS measurements. To gain deeper mechanistic insight experiments conducted with complementary methods will be required.

Referee #3:

In this manuscript, Dufloo et al. analyses the role of complement dependent cytotoxicity (CDC) in presence of new generation of neutralizing antibodies. They show that the potent neutralizing monoclonal Abs was unable to display complement dependent cytotoxicity (CDC) when primary cells are used. These results are similar to those previously published for other anti-HIV Abs. Moreover, CDC was observed following knock down of CD59 by CRISP-Cas9. These results further confirm previous published results indicating that CD46, CD55 and CD59 downregulate complement and that this mechanism is used by HIV to escape from CDC. These results are central and therefore should be presented as main figure in the manuscript and not just in figure S4 as supplementary data.
Moreover, the authors show that CDC was observed on Raji cell lines as previously observed for other anti-HIV Abs. However, as this cell line do not express CD59, this activity may not be physiologically relevant. This should be more precisely stated in the manuscript
What is the meaning of "complement activation" use in the title and all over the manuscript? Is this term used for the C3 expression induced in the presence of Abs. Authors should more clearly indicate to which complement mechanism they refer to.
Overall, according to previous knowledge on CDC, the manuscript as presented here is quite misleading. Authors should modify the presentation of their results and more clearly indicate their finding i.e., CDC only in Raji in cell lines (line 5 of the abstract) and not in primary cells as previously published for other anti-HIV antibodies.

The most surprising results are shown in Figure 5 proposing the disappearance of infected cells by complement. This figure rises however a lot of questions concerning the methods used:
1) Only half of sorted infected cells express Gag at day 1. Is the other half of cells non-infected cells susceptible to get infected? If so, this non infected cells may become infected by newly produced virus and the decreased Gag + cells detected at day 6 with the Nab 10-1074 may be the results of its neutralizing effect. In this case, the inhibitory effect at day 6 may not be due to "disappearance" of infected cells due to complement as proposed by the authors, but to neutralization of newly infected cells by this Nab after 6 day of culture. An antiviral compound should be added to define if infected cells detected at day 6 are newly infected cells or residual infected cells that persist after 6 days of culture

2) In fact, infected cells are quite fragile. Is the staining of primary cells performed on living cells after life dead staining? Material and methods refer to life dead marker for Raji cells but not for primary cells. Staining should be performed on living cells as author mention disappearance of infected cells. Is this disappearance due to the destruction of infected cells?

3) What is the toxicity of NHS compared to HIHS? According to dot plots of figure 5B, higher number of dots (cells) are detected in HIHS compared to NHS for both Ab tested at day 6. This suggest increased cell replication in HIHS compared to NHS. Indeed the concentration of serum 50% is quite high. It is known that high serum concentration is toxic for the cells cultured in vitro, although in vivo, cells are in 100% serum. This is a real experimental limitation for the study of the role of complement. Please comment

4) In HIHS cultured cells, the dot plots are very condensed, and the separation between infected and non-infected cells is not obvious. Again, treatment with potent anti-HIV compound may help draw the gates and to delimitate the infected versus non-infected cells.
5) Finally, the increased disappearance of cells infected with ΔVpu is not obvious. Only day 3

showed differences between WT and ΔΔVpu (Figure 5D). Was this experiment repeated with different cell donors? Again virus replication should be determined for this two viruses (with treatment with an anti-HIV compound) as a difference in virus replication may also explain this results
In figure 5, experiments should be perform to verify that the decreased infected cells observed at day 6 is not due to the neutralizing effect of 10-1074. In that case, complement may not be involved

Additional comments :
Authors refer to primary HIV-strains. However, the viruses used on primary cells had indeed primary envs but were produced by transfection of 293T cells. Again these viruses may not contain the same ratio of CD46, CD55 and CD59 after budding as if they were produced on primary cells. This should be clearly mentioned in the manuscript as it may impact on CDC activity.
In Figure 4D, C3 deposition is very low for 10-1074 treatment with WT virus. Indeed MFI of 10-1074 is also very low figure 3D with a mean of less than 2000. In figures 4E and 6C, D, and E the scales are higher with 50 000, 40 000, and 10 000. Also figure 7, the MFI of C3 expression is low figure 7B (<1 600) and high (10 000) figure 7D. Therefore, the relevance of this low C3 expression with WT viruses is questionable. These scale differences should be clearly mentioned. Maybe the same scale should be used for C3 deposition all over the manuscript!

Authors propose a correlation between env expression, Ab binding and C3 expression. This, however, does not seem to be the case for mAb m66.6 Figure 1 on Raji cells with poor Ab binding, relatively high C3 expression but no CDC. Figure 4, CDC was increased with increased expression of envs on Raji cells. Does this also lead to increased C3 deposition? Noteworthy, previous studies showed that C3 bind poorly to envs. Please comment and precise how env may modify C3 expression and CDC function.

As CD59 is central for CDC, analyzing the expression of CD59 in these different experiments, primary cells infected with WT or ΔΔVpu or Nef would have been of great interest.

1st Revision - authors' response 30 September 2019

Referee #1:

Dufloo et al. describe a series of experiments in which they measure the deposition of C3 on cells expressing HIV ENV (either ectopically or because of viral infection) after incubation with anti-ENV antibodies. Despite C3 and C5b-9 deposition being enhanced by one of the antibodies, no CDC killing was observed. The reasons for this are not clear. However, there is evidence that C3 deposition may accelerate the disappearance of infected cells through an unknown mechanism. The possible control of HIV infection by complement is an important area of study, in particular when induced by bNAbs. This is because the production of bNAbs through vaccination remains a major international goal. The data presented by the authors are therefore important to consider and are of broad interest to the field.

We thank reviewer #1 for appreciating the importance of our work. We have provided an extended analysis of the role of C3 deposition in Env-expressing or HIV-infected CD4+ T cells. We have further described how both viral proteins (Env, Nef and Vpu) and cellular proteins (CD59) modulate CDC in HIV-1 infected cells. We thus provide mechanistical explanations about why infected lymphocytes are poorly sensitive to CDC. We have modified the text to further highlight our findings.

For instance, lines 104-106 of the new version:

"The viral accessory proteins Vpu and Nef down-modulate Env levels and C3 deposition at the plasma membrane, whereas the cellular protein CD59 prevents rapid lysis of infected cells."

and lines 347-350, in the discussion:

"In the presence of these bNAbs, HIV-1-infected cells display C3 deposition and MAC formation, but are not rapidly killed. CDC of infected lymphocytes is prevented by the low levels of Env at the surface, by the constituvely expressed molecule CD59, and by the viral accessory proteins Vpu and Nef."

We also modified the abstract. It now reads (lines 27-31):

"Primary CD4 T cells infected with lab-adapted or primary HIV-1 strains and treated with bNAbs are susceptible to C3 deposition but not to rapid CDC. The cellular protein CD59 and viral proteins Vpu and Nef protect infected cells from CDC mediated by bNAbs or by polyclonal IgGs from HIV-positive individuals."

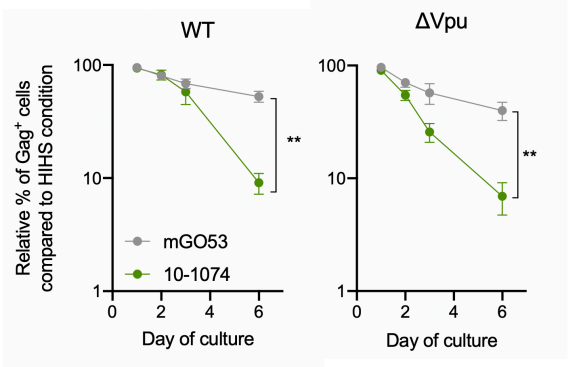
We report that C3 deposition may accelerate the disappearance of infected cells. To our knowledge, this is the first report that C3 deposition in the absence of detectable CDC may trigger an antiviral activity. The underlying mechanism is not characterized yet. This question deserves an entire new study. However, our working hypotheses are exposed in the discussion (lines 391-400):

"We further report that this non-lytic complement deposition leads to an accelerated disappearance of infected cells within a few days of culture. This may be due to a sensing of complement at the cell surface, leading to intrinsic signaling and modulation of cellular activation, metabolism or growth kinetics [6–8]. Complement deposition may also trigger additional effects on infected cells, such as reducing infectivity of released virions [64] or increasing susceptibility to clearance by phagocytic cells [65]. Complement-dependent signaling through CD46 promotes a metabolic switch, with activation and contraction of T cell responses [66,67]. The mechanisms by which antibody-mediated complement deposition modifies the fate of HIV-1-infected lymphocytes will deserve further experimentation."

Furthermore, even if the mechanism by which complement decreases the frequency of infected cells is not detailed, we observed that the disappearance of infected cells occurs faster with a ΔVpu virus. This confirms that Vpu may protect infected against the activity of the complement.

This result is available Fig. 5D:

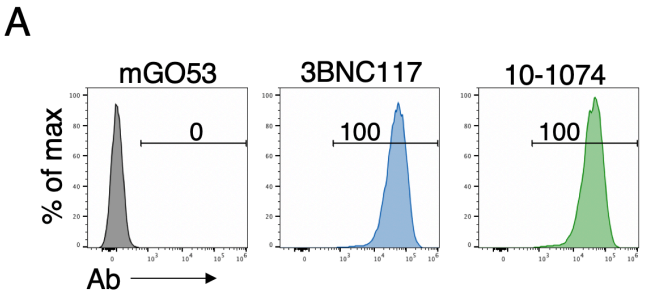
D



The following could be addressed in a revised manuscript and would help solidify the results:

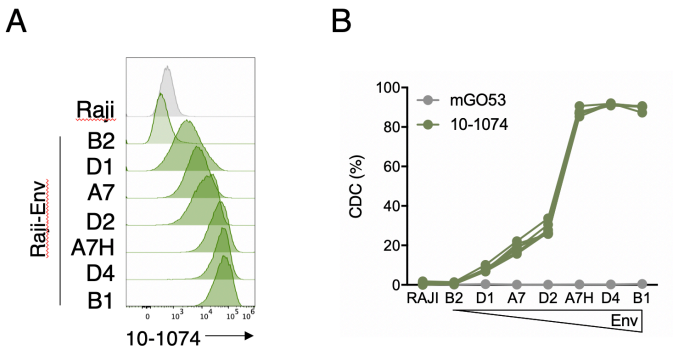
1. Figure 1B Raji B cells express Fcgamma receptors, which will bind to antibodies via their Fc. Repeating the experiment in cells that aren't expressing ENV would be a useful control.

Raji cells indeed express Fc Receptors. To avoid potential biases, we compared all tested antibodies to a control irrelevant antibody with the same isotype (mGO53). As now shown in Fig. 1B, mGO53 does not bind to Env-expressing Raji cells.



Moreover, the experiment requested by reviewer #1 is presented in Fig. 3A, showing that the 10-1074 bNAb does not bind to parental Raji cells. Fig. 3B further shows that 10-1074 does not induce CDC in parental Raji cells.

Figure 3A and B:

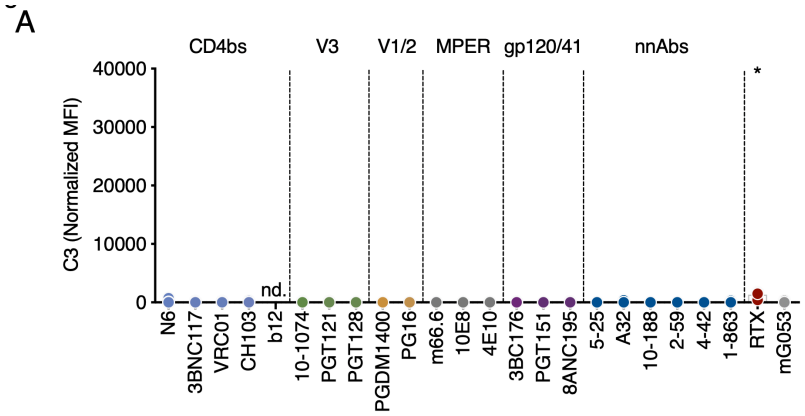


These results are now depicted lines 213-216:

"We generated Raji-Env clones stably expressing various levels of viral glycoproteins, as observed by the increase in the MFI of 10-1074 binding (Fig 3A). Of note, 10-1074 does not bind to parental Raji cells (Fig 3A)."

2. Figure 1D A very high concentration of anti-C3 antibody was used in this experiment (only 1:50 dilution), which makes the possibility of cross-reaction with antibodies on the surface of the Raji cells a concern. To verify these results, the experiment should be repeated using heat inactivated serum.

We agree with reviewer #1 that a high concentration of staining antibody may increase background. The requested data with HIHS are now available in the supplementary Fig. 1A:

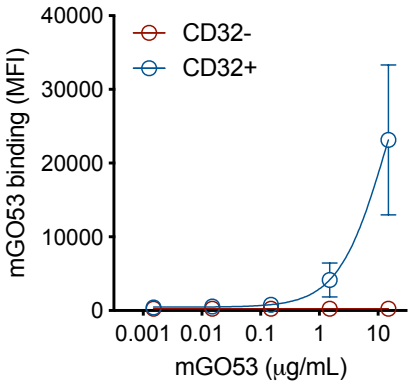


The corresponding section reads (lines 141-142):

"No detectable C3 deposition was observed in cells treated with antibodies and HIHS (Appendix Fig S1A)"

3. Figure 3 T cells also can express FcgRs; I'm therefore surprised that there was no binding measured with the control antibody mGO53. Is the control antibody properly folded and functional? Is it capable of binding antibody receptors?

We agree with the reviewer that an appropriate antibody folding is required for binding to Fc Receptor. All our antibodies were tested for Fc binding. Please find below for instance a dose-response curve of our control antibody mGO53 binding on a CD4 T cell line (Jurkat) over-expressing or not the CD32 FcR. mGO53 binding is detectable at a high concentration only on cells over-expressing CD32, demonstrating its ability to be recognized by Fc receptor. This experiment strongly suggests that the control antibody is correctly folded. Moreover, this antibody was produced simultaneously with the other antibodies, which we know are correctly folded because they recognize their cognate Env antigen.



4. Figure 3 D&F: It would be helpful to show which specific antibodies did not give statistically significant data and/or mention this in the text. For example, it looks like only 10-1074 gave significant C5b-9 deposition.

In this figure, only 10-1074 triggered a significant C5b-9 deposition. To improve clarity, we included a statement in all figure legends to explain that non-significant statistics are not depicted. It reads:

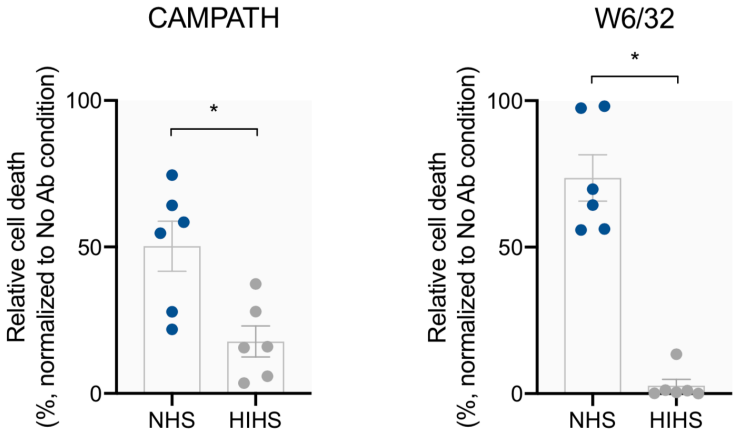
"only significant comparisons are depicted"

5. Supplementary Figure 3B: anti-CD52 triggers ADCC in addition to CDC, so use of this control may not indicate that it is complement killing the cells in this experiment. This should be addressed

by repeating with heat-inactivated serum. It's important that these positive controls can be relied upon to demonstrate that CDC of the cells is possible.

It is unlikely that ADCC may occur in our experimental system, since CD4+ T cells are purified and most of NK cells, which are required for ADCC, have been removed. However, to address this request, we added results of CDC with anti-CD52 antibody in presence of NHS or HIHS in the novel supplementary Fig. 3B. We also included experiments with an HIHS control for CDC of W6/32. As visible in supplementary Fig. 3B, lysis in presence of NHS is significantly higher than in presence of HIHS, demonstrating the ability of anti-HLA-A/B/C W6/32 and anti-CD52 CAMPATH antibodies to trigger CDC in primary CD4 T cells.

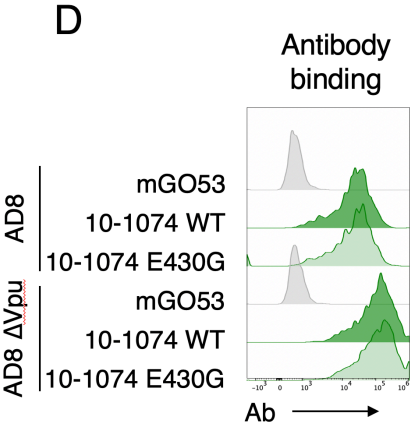
Novel supplementary Fig. 3B:



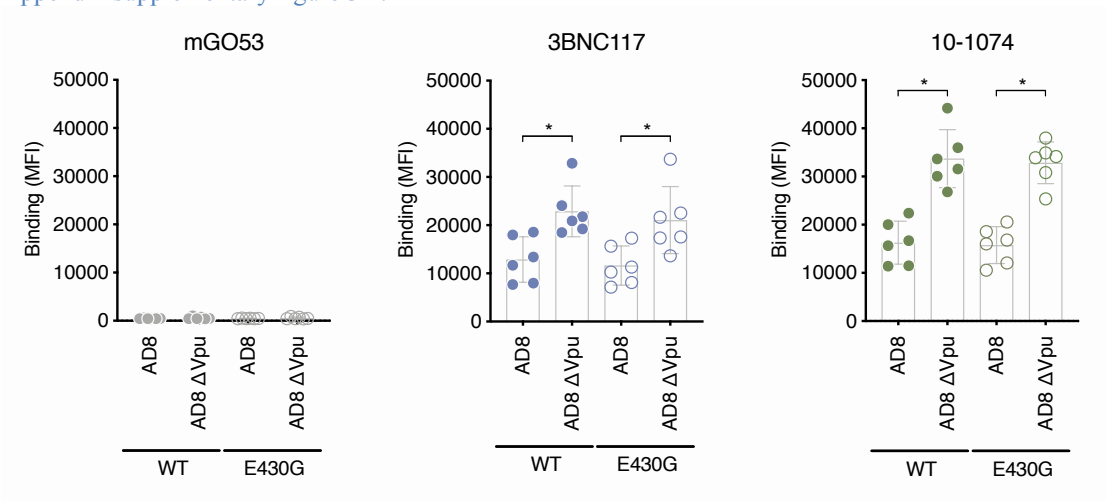
6. Figure 4. The data indicate that Vpu limits complement deposition but this may simply be because Vpu limits the amount of Env expressed at the cell surface. The amount of Env expressed on the cell surface after infection with wild-type veruses deltaVPR virus should be compared and correlated with complement deposition.

We agree with the reviewer that Vpu activity is due to its ability to limit surface Env levels. This is our interpretation of these results. The levels of Env at the surface of WT and ΔVpu infected cells are now visible in Fig. 3D. We also included two novel supplementary figures. Supplementary Fig. 3A summarizes Env expression in WT and ΔVpu virus-infected cells in 6 experiments. Supplementary figure 3B is a correlation between the intensity of anti-Env binding at the surface of infected cells and complement deposition.

Figure 3 D:



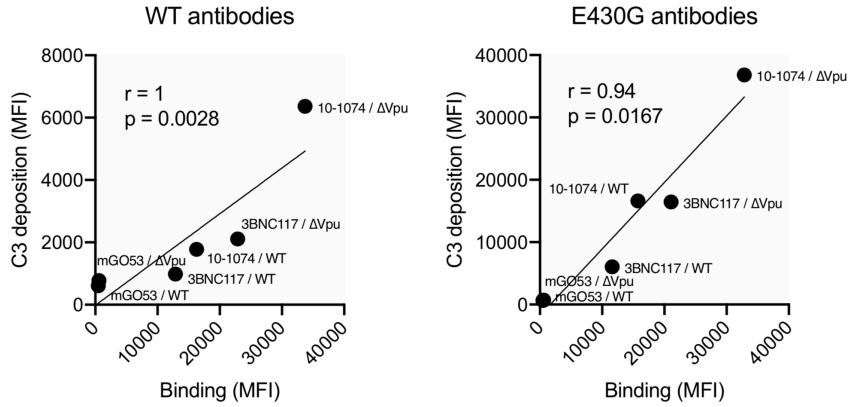
Appendix supplementary figure 3A:



The corresponding result section now reads (lines 231-233):

"As expected, Vpu deletion increased binding of both antibodies at the cell surface (Fig 3D and Appendix Fig S3A). The E430G mutation did not influence antibody recognition of infected cells (Fig 3D and Appendix Fig S3A)."

Appendix supplementary Figure 3B:



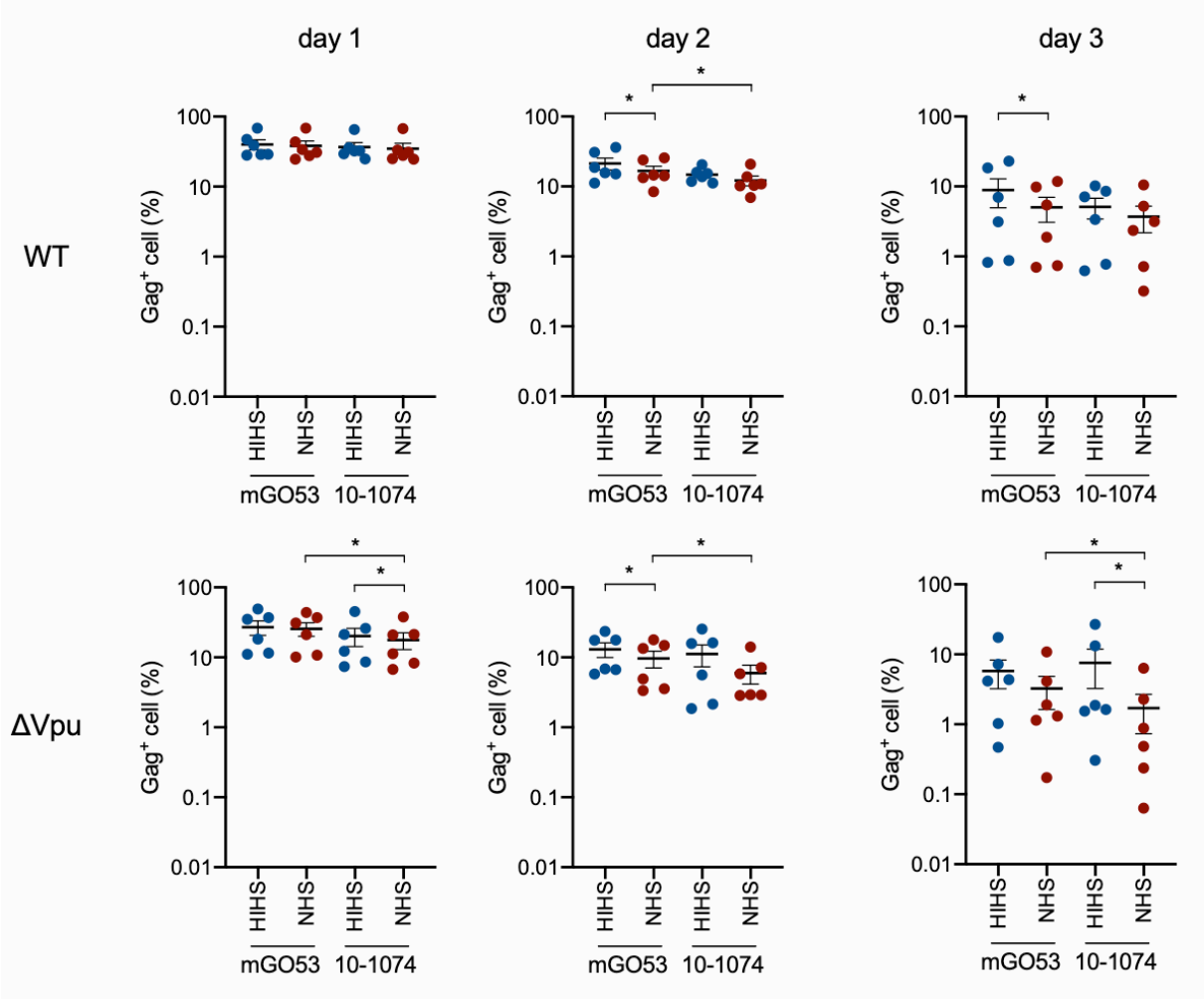
The corresponding result section now reads (lines 239-240):

"Antibody binding correlates with C3 deposition (Appendix Fig S3B)"

7. Figure 5: I don't think the FACS plots are really necessary. The data should instead be graphed (as in 5C) and statistics applied to each day. This will allow significance to be more easily assessed.

We respectfully disagree. We believe that displaying raw data is important to provide all possible information to the reader. However, as requested, we added a novel supplementary figure 5 on which the data are plotted, and statistics were applied for each time point.

Novel appendix supplementary Fig. 5:



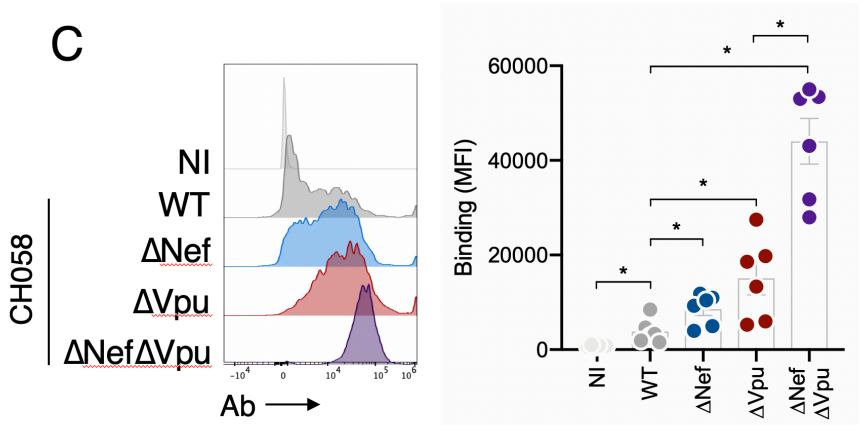
The corresponding result section now reads (lines 274-276):

"The frequency of HIV-1-infected cells was then assessed in living cells (live/dead negative) at days 1, 2, 3 and 6 (Fig 5 and Appendix Fig S5A)"

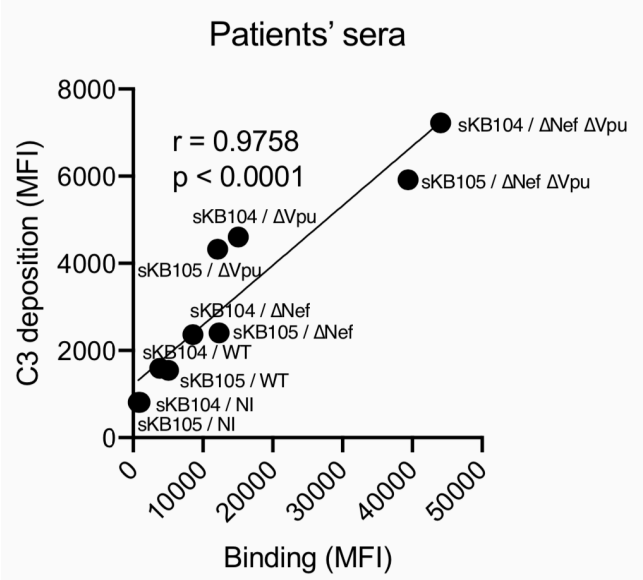
8. Figure 7: The mechanism by which Vpu and Nef limit C3 deposition should explored. Is this simply by limiting ENV expression? Again, the expression of ENV should be compared under the different conditions and correlated with C3 deposition.

We thank reviewer #1 for helping us to improve this important figure. The amount of Env available for recognition by antibodies is now available in Fig. EV2C,D. This novel analysis reveals that antibody binding (indicative of Env expression/accessibility) correlates with C3 deposition. The correlation between antibody binding and C3 deposition is now available in a novel supplementary Fig. EV2B. In line with reviewer comment, our interpretation is that Vpu and Nef limit Env levels or accessibility to antibodies, subsequently limiting C3 deposition. We believe that Fig. EV2 and its associated appendix supplementary Fig 7 now provide a mechanism by which Vpu and Nef regulate C3 deposition by anti-Env antibodies.

Fig. EV2C:



Novel appendix supplementary Fig. 7B:



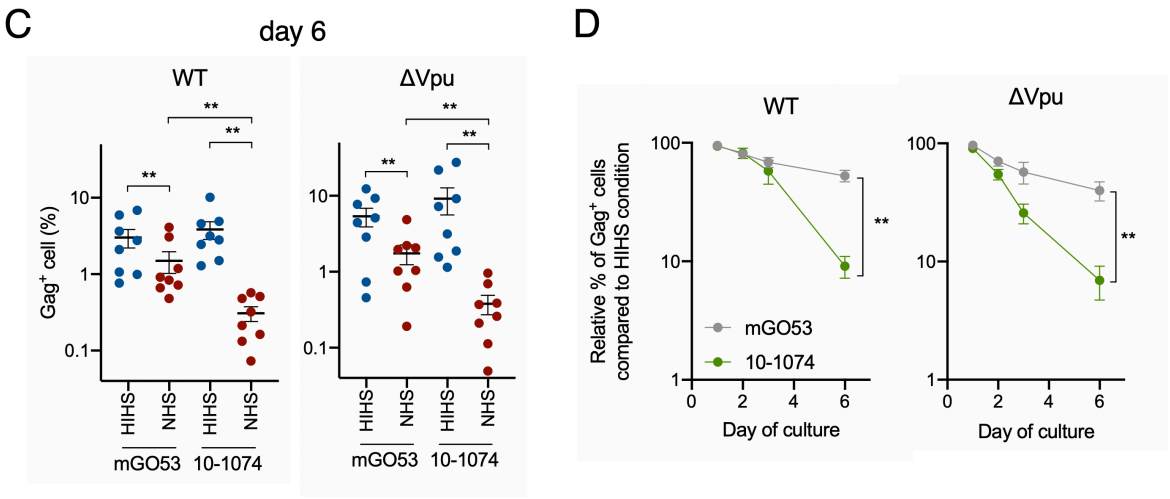
The corresponding result section now reads (lines 333-336):

"Complement deposition correlated with antibody binding, with higher C3 deposition on ΔNef and ΔVpu-infected cells compared to the WT virus. This phenomenon was exacerbated on ΔNefΔVpu-infected cells (Fig EV2D and Appendix Fig S7B)"

The findings of this study are slightly because no CDC of infected cells was demonstrated for anti-ENV antibodies. Thus it is difficult to know how important Vpu or Nef antagonism is in limiting C3 deposition. Possibly this could be partially addressed by repeating the experiments in Figure 5 using viruses where Vpu or Nef has been deleted and determine whether this alters persistence of infected cells in the presence of the anti-ENV antibodies.

A virus lacking Vpu is now included in Fig. 5. These data show that the decrease of the fraction of Gag^+ cells was more rapid with ΔVpu than with WT-infected cells (Fig. 5C and D), demonstrating that Vpu antagonizes complement antiviral activity.

Fig. 5C and D:



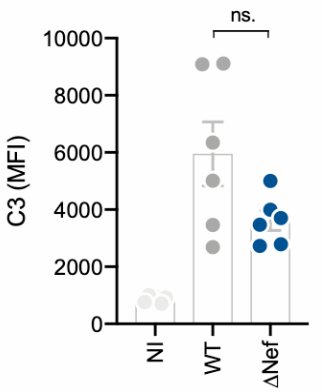
The results are mentioned in the text (lines 271-273):

"Enriched WT or ΔVpu NLAD8-infected cells (up to 90% of Gag$^+$ cells) were then cultured with 10-1074^{E430G} or isotype control in the presence of NHS or HIHS"

And (lines 284-285):

"The decrease of the fraction of Gag$^+$ cells was more rapid with ΔVpu than with WT-infected cells (Fig 5D)

Moreover, we observed that Nef deletion does not increase C3 deposition induced by 10-1074 on HIV-1-infected cells (see below).



Referee #2:

Dufloo et al describes how a number of broadly neutralizing HIV-1 antibodies activates the complement system on cells engineered to express high levels of ENV and on primary CD4+ cells infected with HIV-1. Understanding how antibody effector functions, including complement, contributes to protection against HIV-1 is important to inform on future therapeutic strategies and vaccine design. The authors show that only a subset of bNAbs activate complement and that complement is not potently activated due to low level of ENV expression which are regulated by Nef and Vpu. Furthermore, they show that expression of CD59 on CD4+ T-cells counteract CDC. The conclusions regarding complement activation are supported a large number of solid experiments and agree with the current concepts of antibody mediated complement activation. The text and figures are well composed and easy to follow even for the non-expert. Whereas the presented work could be of significant interest for an HIV oriented audience it is possibly too specialized for a broader audience including complement focused readers.

We thank reviewer #2 for his/her positive comments. We respectfully disagree with the appreciation of the broadness of our study. We believe that understanding how antibodies use the complement goes well beyond the HIV field. The increasing clinical use of antibodies to treat inflammatory, auto-immune, cancer and infectious diseases warrants a careful analysis of their activity.

Minor issues

1. It would be helpful if background on Nef and Vpu regulation of ENV expression was presented in the introduction or at first mention under results as this is important for the broader audience.

We agree with the reviewer that additional background on HIV is required for a general audience. We extended the description of Vpu and Nef activity at their first mention in the result section.

The sentences now read (lines 225-228):

"The viral accessory protein Vpu eliminates the cellular protein tetherin/BST-2 from the membrane of infected cells. Tetherin retains budding viral particles at the cell surface, limiting viral spread and increasing recognition of infected cells by antibodies and ADCC [49–51]."

And (lines 319-326):

"In most individuals, the anti-HIV-1 polyclonal antibody response consists of non- or poorly-neutralizing antibodies that preferentially recognize non-functional or "open" conformation of Env (i.e. bound to the receptor CD4) [53–55]. The viral protein Nef is an infectivity factor that down-regulates various cellular proteins from the surface of infected cells, including CD4, MHC-I and SERINC3/5 [56]. Nef also modulates Env surface levels [57]. By promoting CD4 internalization, Nef limits Env-CD4 interactions at the membrane, thus decreasing recognition by nnAbs and ADCC [48]. Nef synergizes with Vpu to counteract ADCC-mediated killing of infected cells [49,58]."

2. It could strengthen the manuscript if the discussion considered whether combinations of bNAbs could increase CDC (as observed for antibodies against EGFR). Is the amount of ENV on infected CD4+ cells simply too low to effectively to activate complement no matter what antibody that is used?

Indeed, combining antibodies may help reaching sufficient complement deposition to trigger lysis. We included a reference on the work performed with EGFR antibodies and discussed the consequences for HIV following reviewer suggestions. It reads (lines 373-375):

"Combining antibodies may increase complement activation as previously observed for anti-EGFR antibodies [62]. How bNAbs recognizing non-overlapping epitopes may synergize to promote CDC will deserve further investigation."

3. The current manuscript very much appeals to an HIV oriented audience, whereas the complement perspective is less well covered.

We respectfully disagree and believe that our work is appealing not only for HIV specialists, but also for a general audience interested on understanding the mechanisms of action of therapeutic antibodies. Moreover, the description of the role of complement provides novel insights into the interaction of the innate immune system with virus-infected cells. Our main general findings can be summarized as follows:

- We provide the first characterization of the ability of a large panel of anti-HIV bNAbs and nnAbs to trigger the complement cascade at the surface of infected cells.

- We demonstrate that infected CD4+ T cells are resistant to CDC and decipher the underlying mechanisms.

- We provide the first demonstration that the E430G mutation in the Fc domain of an IgG promotes complement activity against an infectious agent.

- The interest to non-lytic complement activation (i.e. sublytic or intracellular complement activation) is growing in the literature. For instance, it was recently reported that non-lethal complement attack activates the inflammasome (Triantafilou et al, J. Cell Science 2013), may favorize cancer progression (Towner et al, J. Biol. Chem., 2016), or modulates cell metabolism (Arbore et al, Nat. Com., 2018 & Hess et al, Immunity 2016).

- The study of complement activity in the presence of different levels of antigens at the plasma membrane is of critical importance in many fields, including autoimmune, inflammatory diseases, cancer and other infectious agents.

We have modified the introduction and discussion to emphasize these elements:

Line 102: "The Fc mutation E430G increases complement activity of HIV-1 bNAbs."

Line 391-399: "We further report that this non-lytic complement deposition leads to an accelerated disappearance of infected cells within a few days of culture. This may be due to a sensing of complement at the cell surface, leading to intrinsic signaling and modulation of cellular activation, metabolism or growth kinetics [6–8]. Complement deposition may also trigger additional effects on infected cells, such as reducing infectivity of released virions [64] or increasing susceptibility to clearance by phagocytic cells [65]. Complement-dependent signaling through CD46 promotes a metabolic switch, with activation and contraction of T cell responses [66,67]."

Line 407-409: "Furthermore, the E430G mutation enhances the complement-activating capacity of 10-1074 and 3BNC117"

4. Abstract "Here, we show that a subset of broadly neutralizing antibodies (bNAbs), targeting the CD4 binding site and the V3 loop". Already here the non-HIV reader is lost, what is the antigen?

We have modified the abstract which reads (lines 23-24):

"Here, we show that a subset of anti-HIV-1 Envelope (Env) broadly neutralizing antibodies (bNAbs), targeting epitopes in the CD4 binding site and the V3 loop"

5. A better presentation of C3, C3b regulation on cells and how C3b density correlates with C5 convertase and terminal pathway initiation is needed. In fact, what is measured during "C3 deposition" are its degradation products C3b, iC3b.

We agree that the terms "C3 activation" or "C3 deposition" lacks precision considering the various cleaved forms of C3. The antibody we used (Clone 6C9) recognizes C3, C3b and iC3b. We thus chose to use the term "C3 deposition" as we cannot define more precisely which form of C3 is deposited on infected cells. To improve clarity, we included a more precise characterization of the C3 antibody we used. It reads (lines 479-482):

"To measure complement deposition, cells were incubated with anti-C3/C3b/iC3b-APC (Clone 6C9, Cerderlane) or anti-C5b9 (clone aE11, Abcam) biotinylated in-house (see above) followed by streptavidin-AlexaFluor647 (ThermoFisher)."

We also modified the text at the first mention of C3 deposition in the results (line 136-137):

"Activation of C3 was assessed by measuring C3b/iC3b deposition (later referred to as C3 deposition) by flow cytometry after 1h"

6. On p8, the "open conformation of Env" is mentioned, what is this and how does it differ from other Env conformations?

This information has been included in the introduction (line 56-59):

"The HIV-1 glycoproteins gp120 and gp41 assemble as a trimer to form the viral envelope (Env). Recognition of the receptor CD4 reorganizes Env to allow fusion by exposing the fusion peptide of gp41. Env exists in at least two conformations, a native "close" configuration and a CD4-bound "open" conformation (reviewed in [11])"

And in the results (line 320-321):

"open" conformation of Env (i.e. bound to the receptor CD4)"

7. Discussion "Recognition of complement components by CR2 on B cells decreases the threshold of BCR stimulation, favoring an optimal response". CR2 only recognize iC3b and C3dg.

This has been corrected, as stated in the text (lines 417-418):

"Recognition of iC3b and C3dg by CR2 on B cells decreases the threshold of BCR stimulation"

8. Methods: Provide the specificity of the C3 antibody used here for detection

Done, see point 5.

9. Fig 1 legend "the MFI the of no antibody" needs correction

Done

10. Fig S2. The legends says red curve, it is not red.

Corrected

Major issues

1) Given the current understanding of antibody mediated complement activation, the lack of complement activation on cells expressing low amount of antigen (ENV) is to be expected. The obtained results thus support previous observations but also lack novelty. The work presented is very descriptive but lacks mechanistic models at the molecular level that are evaluated by experiments. The most important example of this is the lack of attempt to explain why sublytic MAC formation induce disappearance of infected T-cells.

Some of our results may be seen retrospectively as being expected, but to our knowledge this is the first report of an analysis of a large panel of bNAbs on the complement pathway, and their impact on CDC in different experimental systems.
We respectfully disagree with the reviewer regarding the lack of mechanistic models and novelty for several reasons:

1. As bNAbs overcome the variability of Env, it was not predictable that bNabs will fail to trigger CDC of primary HIV-1-infected CD4 T cells. Moreover, several reports previously proposed that HIV-1-infected cell lines are susceptible to CDC. We believe that our study provides convincing evidence that primary CD4 T cells are not lysed by CDC. We further describe the underlying molecular mechanisms, by describing the combined and independent activities of viral proteins (Vpu and Nef) and cellular proteins (CD59).

2. The overall novelty of our work is based on:
-The identification of V3-targeting antibodies as the best inducers of complement deposition was not predictable based on the current literature.
-To our knowledge, this is the first report of an antiviral consequence of non-lytic complement deposition. Moreover, given that numerous pathogens escape antibody recognition by decreasing the levels of antigens at the surface, it is likely that this non-lytic complement activity may occur in other infectious contexts. Understanding the underlying mechanisms will require an entire new study, which we believe is out of the scope of this report.

The experimental work is very much based on FACS measurements. To gain deeper mechanistic insight experiments conducted with complementary methods will be required.

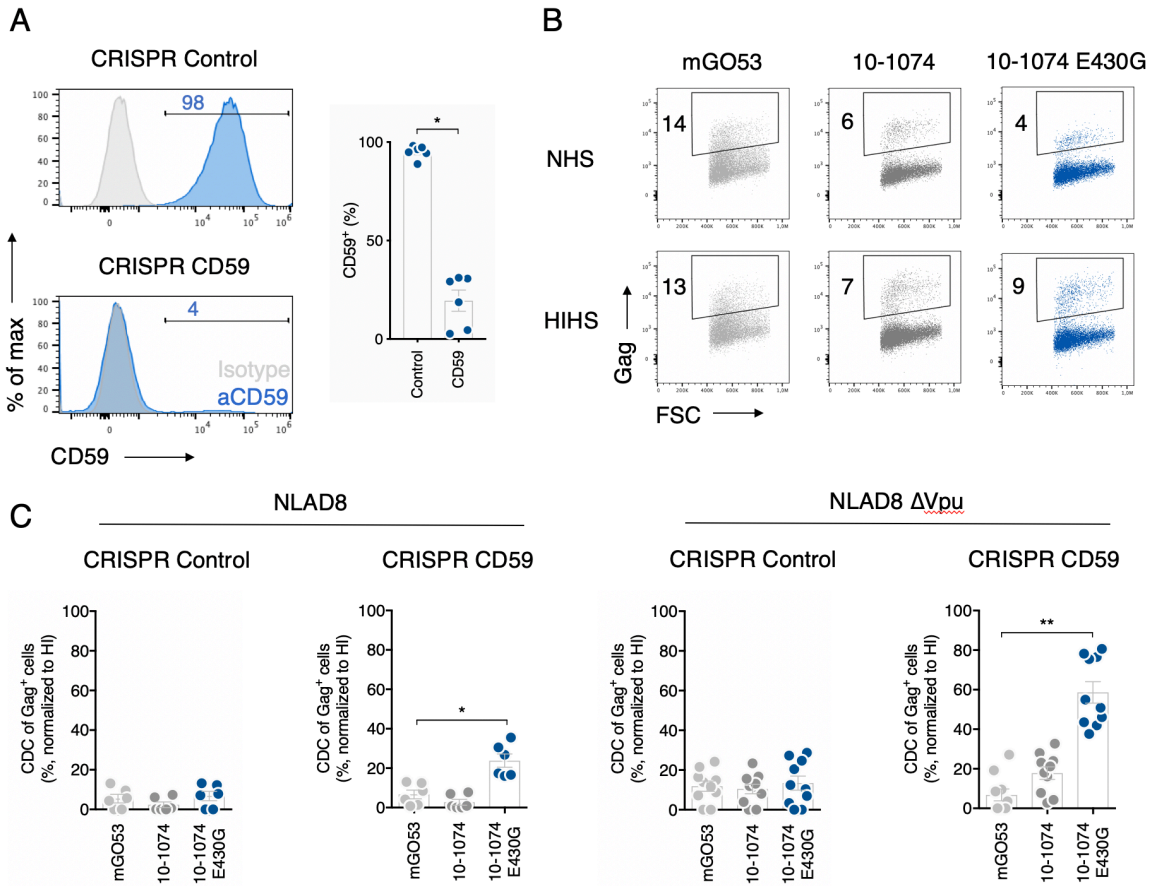
Flow cytometry has the unique ability to provide protein information at the single cell level, which makes it highly valuable compared to other technics and justifies its use throughout the manuscript. Additional technics, such as Western Blot, require more cells and provide information on bulk populations, which is problematic when working with primary cells, in which only a fraction of cells is infected as being the case in our study. Of note, our results in Raji cells were confirmed by live imaging (see Supplementary movie).

Referee #3:

In this manuscript, Dufloo et al. analyses the role of complement dependent cytotoxicity (CDC) in presence of new generation of neutralizing antibodies. They show that the potent neutralizing monoclonal Abs was unable to display complement dependent cytotoxicity (CDC) when primary cells are used. These results are similar to those previously published for other anti-HIV Abs. Moreover, CDC was observed following knock down of CD59 by CRISP-Cas9. These results further confirm previous published results indicating that CD46, CD55 and CD59 downregulate complement and that this mechanism is used by HIV to escape from CDC. These results are central and therefore should be presented as main figure in the manuscript and not just in figure S4 as supplementary data.

We thank reviewer #3 for the review. We have addressed each point carefully and we hope that this novel version will satisfy him/her.

As suggested by reviewer #3, we have now included the figure demonstrating that CD59 blocks CDC in primary CD4 T cells in the main text, as the new Fig. 4:



Moreover, the authors show that CDC was observed on Raji cell lines as previously observed for other anti-HIV Abs. However, as this cell line do not express CD59, this activity may not be physiologically relevant. This should be more precisely stated in the manuscript

Raji cells were used in the first part of the manuscript as a model system to screen antibodies and characterize their ability to trigger C3 deposition and CDC. We agree that the lack of CD59 in these cells needs to be more precisely stated. This is now clearly indicated:

Lines 117-121:

"We chose Raji cells because (i) they lack CD4 and Env is not fusogenic in the absence of CD4, (ii) they do not express CD59, making them higly sensitive to complement lysis, (iii) treatment with the anti-CD20 antibody rituximab (RTX) triggers complement activation in Raji cells [45], providing a convenient positive control."

Lines 176-178:

"Raji-Env cells do not recapitulate the complexity of HIV-1-infected cells as they express high levels of Env and lack other viral proteins. Moreover, they do not express CD59, one key regulator of CDC."

And lines 250-252:

"We observed CDC in Raji-Env cell that lack CD59, whereas CD4 T cells express high levels of CD59 (Fig 4A)."

What is the meaning of "complement activation" use in the title and all over the manuscript? Is this term used for the C3 expression induced in the presence of Abs. Authors should more clearly indicate to which complement mechanism they refer to.

We agree with reviewer #3 that "complement activation" is misleading, as it may correspond to various outcomes. We thus replaced "complement activation" by "complement deposition" in the title and, we are more precise at different places throughout the manuscript.

Overall, according to previous knowledge on CDC, the manuscript as presented here is quite misleading. Authors should modify the presentation of their results and more clearly indicate their finding i.e., CDC only in Raji in cell lines (line 5 of the abstract) and not in primary cells as previously published for other anti-HIV antibodies.

We agree that the absence of CDC in primary CD4 T cells should be more clearly presented. For instance, we changed the abstract (lines 23-26):

"Here, we show that a subset of anti-Envelope (Env) broadly neutralizing antibodies (bNAbs), targeting the CD4 binding site and the V3 loop, triggers C3 deposition and complement-dependent cytotoxicity (CDC) on Raji cells engineered to express high surface levels of HIV-1 Env"

And (lines 27-29):

"Primary CD4 T cells infected with lab-adapted or primary HIV-1 strains and treated with bNAbs are susceptible to C3 deposition but not to rapid CDC."

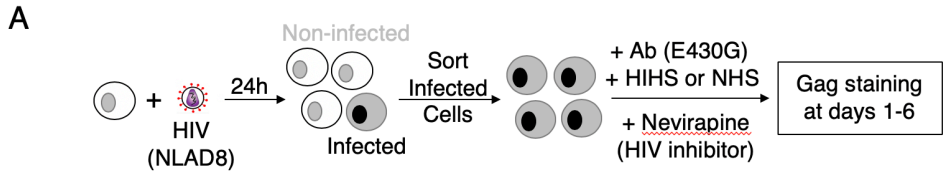
The most surprising results are shown in Figure 5 proposing the disappearance of infected cells by complement. This figure rises however a lot of questions concerning the methods used:
1) Only half of sorted infected cells express Gag at day 1. Is the other half of cells non-infected cells susceptible to get infected? If so, this non infected cells may become infected by newly produced virus and the decreased Gag + cells detected at day 6 with the Nab 10-1074 may be the results of its neutralizing effect. In this case, the inhibitory effect at day 6 may not be due to "disappearance" of infected cells due to complement as proposed by the authors, but to neutralization of newly infected

cells by this Nab after 6 day of culture. An antiviral compound should be added to define if infected cells detected at day 6 are newly infected cells or residual infected cells that persist after 6 days of culture

We fully agree with this comment. Experiments were performed in the presence of Nevirapine. This is now indicated in the main part of the manuscript (lines 273-274):

"Cells were maintained in the presence of the reverse transcriptase inhibitor Nevirapine to prevent viral propagation to remaining non-infected cells."

We modified Fig. 5A to include this information in the cartoon:



2) In fact, infected cells are quite fragile. Is the staining of primary cells performed on living cells after life dead staining? Material and methods refer to life dead marker for Raji cells but not for primary cells. Staining should be performed on living cells as author mention disappearance of infected cells. Is this disappearance due to the destruction of infected cells?

We thank reviewer #3 for raising this point of critical importance. Cells were stained with a Live/Dead reagent to exclude dying cells. We modified the results, the methods and the legend of Fig. 5 to mention that we performed a gating on live cells.

In the results section (lines 274-276):

"The frequency of HIV-1-infected cells was then assessed in living cells (live/dead negative) at days 1, 2, 3 and 6 (Fig 5 and Appendix Fig S5A)"

In the methods section (line 506-508):

"For CD4 T cells, cells were fixed with 4% PFA and stained for intracellular Gag, in some experiments the live/dead fixable aqua dead cell marker (1:1,000 in PBS, Life technologies) was added for 30 min at 4°C before fixation"

In the legend of Fig. 5:

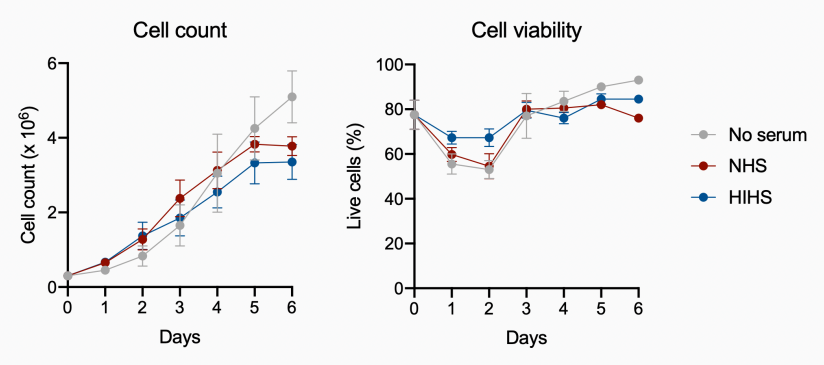
Line 688 "The % of Gag$^+$ cells was measured in live cells by flow cytometry at days 1, 2, 3 and 6."
Line 691: "The frequency of infected cells was assessed by flow cytometry in live cells at days 1, 2, 3 and 6."
Line 693: "Frequency of live HIV-1-infected cells (NLAD8 WT or ΔVpu strain) 6 days after incubation with the indicated antibodies, and with NHS or HIHS."

3) What is the toxicity of NHS compared to HIHS? According to dot plots of figure 5B, higher number of dots (cells) are detected in HIHS compared to NHS for both Ab tested at day 6. This suggest increased cell replication in HIHS compared to NHS. Indeed the concentration of serum 50% is quite high. It is known that high serum concentration is toxic for the cells cultured in vitro, although in vivo, cells are in 100% serum. This is a real experimental limitation for the study of the role of complement. Please comment

As discussed by the reviewer, cells are often exposed in vivo to 100% concentration of serum. Human serum is not expected to be toxic for human primary lymphocytes. However, it may be toxic for immortalized human cells, cells from other species and bacteria/ parasites/viruses. As requested, we tested the toxicity of human serum in cultures of primary human CD4+ T cells. As shown below,

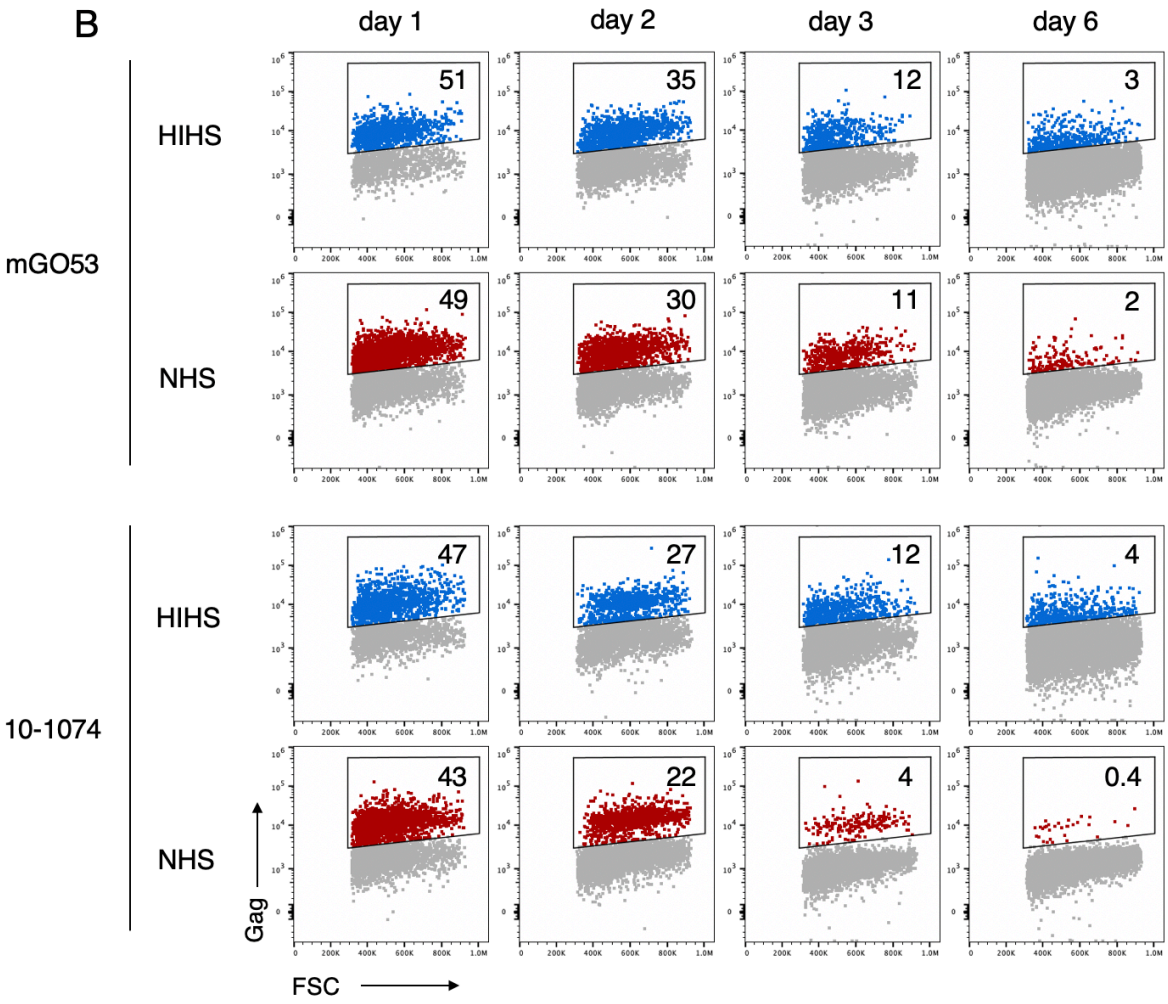
50% of NHS in the culture was not toxic and did not impact cell growth. The results are now mentioned in the text page (line 290-291):

"NHS in the culture was not toxic and did not impact cell growth (Appendix Fig S5B)"
Novel appendix supplementary Fig. 5B:



4) In HIHS cultured cells, the dot plots are very condensed, and the separation between infected and non-infected cells is not obvious. Again, treatment with potent anti-HIV compound may help draw the gates and to delimitate the infected versus non-infected cells.

As explained above, the antiviral compound nevirapine was added in all experiments.
We have magnified the panel of the Figure to render more visible the separation between infected and non-infected cells:



5) Finally, the increased disappearance of cells infected with ΔVpu is not obvious. Only day 3 showed differences between WT and ΔΔVpu (Figure 5D). Was this experiment repeated with different cell donors? Again virus replication should be determined for this two viruses (with treatment with an anti-HIV compound) as a difference in virus replication may also explain this results

The antiviral compound nevirapine was present in the cultures. The experiments were performed in triplicates with cells from 6 independent donors (day 1, 2 and 3). We also performed additional long-term (day 6) experiments with two additional donors, allowing a statistical analysis on 8 different donors at day 6
This is indicated in the legend of Fig. 5C (line 694-695):

"Each dot represents a single donor of CD4 T cells. n=8 donors of CD4 T cells."

As mentioned by the reviewer, the difference between WT and ΔVpu viruses is visible at day 3 (with data from 6 donors of CD4 T cells). We conclude in the text (lines 284-285):

"The decrease of the fraction of Gag$^+$ cells was more rapid with ΔVpu than with WT-infected cells".

In figure 5, experiments should be perform to verify that the decreased infected cells observed at day 6 is not due to the neutralizing effect of 10-1074. In that case, complement may not be involved

As previously discussed, nevirapine was included in the cultures from days 0 to 6. Thus, the virus is not spreading in this system. Moreover, the use of HIHS provides an additional control. Indeed,

comparing 10-1074 and mGO53 both in presence of HIHS and NHS allows to account for any complement-dependent activity of 10-1074 (occurring specifically in presence of NHS) different than neutralization. Thus, the effect observed is most likely not due to neutralization by bNAbs,

Additional comments:

Authors refer to primary HIV-strains. However, the viruses used on primary cells had indeed primary envs but were produced by transfection of 293T cells. Again these viruses may not contain the same ratio of CD46, CD55 and CD59 after budding as if they were produced on primary cells. This should be clearly mentioned in the manuscript as it may impact on CDC activity.

As stated by the reviewer, our viruses are produced by transfection of 293T cells. This widely used technique allows reproducible virus preparation, as it avoids accumulation of mutations observed in viruses produced by replication on susceptible cells. We agree that "primary" refers to the origin of the transfected virus.
In our experiments we exposed primary CD4+ T cells to 293T-produced viruses for 4h and washed unbound viruses. We then waited for up to 2-3 days to perform experiments on infected cells. Therefore, infected cells produce novel viral progeny exposing physiologically relevant amounts of CD46, CD55 and CD59.
To avoid any confusion, we modified the method section, lines 779-782, which now reads:

"Cells were infected for 4h in the presence of HEPES and DEAE/Dextran, washed to remove unbound viral particles and cultured in presence of IL-2 (50 IU ml^{-1}) for up to 2-3 days before performing further experiments."

In Figure 4D, C3 deposition is very low for 10-1074 treatment with WT virus. Indeed MFI of 10-1074 is also very low figure 3D with a mean of less than 2000. In figures 4E and 6C, D, and E the scales are higher with 50 000, 40 000, and 10 000. Also figure 7, the MFI of C3 expression is low figure 7B (<1 600) and high (10 000) figure 7D. Therefore, the relevance of this low C3 expression with WT viruses is questionable. These scale differences should be clearly mentioned. Maybe the same scale should be used for C3 deposition all over the manuscript!

We agree with the reviewer that the MFI of C3 deposition varies depending on the conditions and may be sometimes low. The variations observed in different figures are also linked to the experimental conditions. To avoid misleading interpretation of the data, we have corrected Fig. 3E and 6C (now EV1C), E as well as Fig. 6D (now Fig. EV1D) and 7D (now Fig EV2D) to display the same scale for C3 deposition between figures. When needed, (in Fig 2D and EV2B) where it was important to visualize statistical differences, we maintained a specific scale. The text has been modified accordingly (lines 538-539):

"Scales were adjusted in each graph to highlight statistically significant differences."

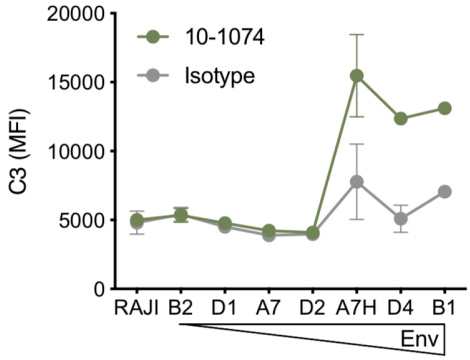
Authors propose a correlation between env expression, Ab binding and C3 expression. This, however, does not seem to be the case for mAb m66.6 Figure 1 on Raji cells with poor Ab binding, relatively high C3 expression but no CDC. Figure 4, CDC was increased with increased expression of envs on Raji cells. Does this also lead to increased C3 deposition?
Noteworthy, previous studies showed that C3 bind poorly to envs. Please comment and precise how env may modify C3 expression and CDC function.

We agree that m66.6 is out of the correlation between antibody binding, C3 deposition and CDC in Raji-Env cells. This is indeed a puzzling observation that will deserve further investigation. m66.6 is a peculiar antibody known to be highly polyreactive against self and non-self antigens (Zhu et al., 2011). This is now briefly stated in the text (lines 145-147)

"Of note, m66.6, an anti-MPER antibody known to be polyreactive against self and non-self antigens [46], displayed C3 deposition in the absence of CDC."

A detailed analysis of the mechanisms underlying the effect of this outlier antibody is out of the scope of our study.

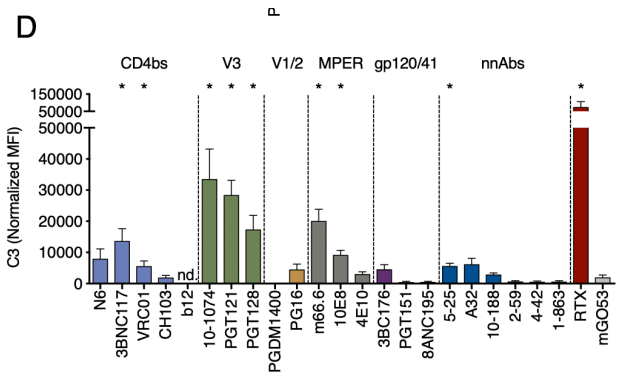
As requested by the reviewer, we analyzed the relationship between C3 deposition and Env levels. As visible in the figure below, C3 deposition increased with surface Env levels:



These data are now included in the corresponding result section, which reads (lines 217-218):

"*The efficacy of C3 deposition and CDC increased with the surface levels of Env (Fig 3B and not shown"*

Concerning C3 binding to Env, there is indeed a literature reporting a direct activation of complement by Env. However, we did not observe C3 deposition with the control antibody mGO53 (Figure 1D), suggesting that this is not the case in our experimental system.



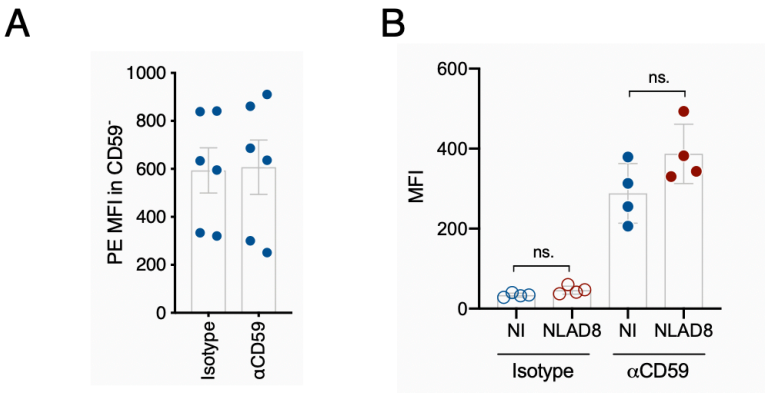
This is now clearly stated in the text (lines 141-143):

"No detectable C3 deposition was observed in cells treated with antibodies and HIHS (Appendix Fig S1A) or in the absence of anti-Env antibodies (Fig 1C and D)."

As CD59 is central for CDC, analyzing the expression of CD59 in these different experiments, primary cells infected with WT or ΔΔVpu or Nef would have been of great interest.

We agree that CD59 is central for CDC regulation. The levels of CD59 expression by non-infected and HIV-infected cells are now presented in the supplementary Fig. 4.

Appendix Figure S4:



And discussed in the text (lines 262-263):

"Of note, HIV-1 infection did not modulate CD59 surface levels (Appendix Fig S4B)

2nd Editorial Decision 4 November 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, all three referees have some remaining questions and suggestions to improve the manuscript I ask you to address in a final revised version. Please also provide a detailed point-by-point-response addressing these.

Further, I have these editorial requests:

- We can publish the final revised paper either as short report, or as full article. Presently, there are 5 main figures and 2 EV figures, plus an Appendix. In this form, we could proceed with this as a report. However, for a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Otherwise, we could expand this to a full article by moving data from the Appendix to the main manuscript. We could accommodate more main figures (up to 8) and up to 5 EV figures. Thus, I wonder if the data shown in the figures of the Appendix could be moved to the main or EV figures, and the Appendix would then only contain the tables. Please decide for one of these options. For more details please refer to our guide to authors: http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- In the Appendix a legend to a Supplementary Fig. 7 is present, but the Appendix does not contain one. Please check.

- The Appendix items are incorrectly named. Please name these 'Appendix Figure S#' and 'Appendix Table S#', and call these out accordingly in the manuscript text.

- Please provide the final Appendix file as pdf.

- Please name the movie file Movie EV1, and change the call-outs accordingly. Please provide a legend for the movie as text file, ZIP it together with the movie file, and upload this to the final manuscript files.

- It seems the same datasets are shown in Fig. S2B and 2C (labelled NHS in 2B; NI in 2C). Please check or explain (also in the respective legends).

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the

modifications done. If figures are moved from the Appendix to the main manuscript, please check that similar issues are addressed in their new legends.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #1:

The manuscript form Dufloo et al. was largely improved in this new version. Most of the concerns have been taken into consideration. These modifications largely increases the comprehension of the field and put forward the novelty of the results compared to previous published data. However, the data showing apparent disappearance of infected cells in the presence of complement are doubtful.

First, what is the rational for enrichment of CD4 T infected cells by magnetic depletion of CD4high non infected cells for the protocol of analysis of disappearance of infected cells? Did author verify that the depletion of CD4+ cells are not infected? It was described that CD4 downregulation was less effective on CD4 primary cells compared to cell lines. Moreover, it is known that magnetic beads depletion retain dead cells. This may explain the unexpected low level of cell death cells detected over the 6 days of culture (supplemental figure 5B, 80% living cells). It is also known that unspecific binding via Fc domain of Abs may occur during depletion strategies. These different experimental caveats may differentially impact the percentage of infected cells recovered for the different treatment (NHS, HIHS, with Abs...). It would be of interest to verify that the protocol of infected cell enrichment used for this manuscript do not interfere with the differential detection of infected cells according to the treatment. Also, please add the detailed protocol for detection of disappearance of infected cells in the Mat and Method section.

Second, the dot plot figure 5 B suggest that cells (number of dots) increased drastically at day 6 in the presence of HIHS in mGO53 and 10-1074 treated cells, but this doesn't seem to be the case in the presence of NHS, with fewer dots, especially when cells are treated with 10-1074. These plots contradict the results of supplementary figure 5 showing continuous increased cell count up to day 6 in presence of NHS or HIHS. Is this cell count corresponding to cells isolated after CD4 infected T cell enrichment?

Third, because of the higher number of cells analyze in HIHS and NHS/GO53 treated cells at day 6, the percentage of gag positive cells may be overestimated compared to NHS/10-1074 treated cells. Indeed the separation between gag+ and gag - is not easy to define, and the gate may need to be heightened. To finely tune the gag positive cells, controls without gag staining should be shown. It would also be of interest to show plot with lower number of cells analyzed similarly as 10-1074 treated cells to better define the gates.

Fourth, the disappearance of infected cells with WT virus is not at all obvious at day 1, 2 and 3 (supplemental figure 5A). With delta-Vpu infected cells, the % of gag+ cells is lower only for one point below 0.1 corresponding to 1 donor day 3. It would have been of interest to label each of the n=6 experiments to identify the corresponding dots for each separate experiment. To compare the role of complement in WT and delta-Vpu infected cells, the % of infected cells recorded with these two viruses should be mentioned.

Fifth, a decreased % of gag expressing cells is systematically detected in cells treated with mGO53 control Ab in the presence of NHS compared to HIHS. This suggest that NHS has a direct inhibitory effect on infected cells. An additional control without Ab should be added to estimate the relative

contribution of mGO53 Ab compared to complement alone. Does this inhibition means that complement has a direct effect on infected cell disappearance? Is this inhibition due to additional factors present in the NHS that is inactivated in HIHS?

Additional comments:
EV1A: The % of Gag infected cells seems extremely and unusually high for primary CD4 T cell infection with primary virus, even higher as in figure 5B where cells were infected with AD8 and enriched. Is this high infection due to DEAE/Dextran treatment? Did authors enriched infected cells by CD4 high magnetic depletion? This high rate of infected cells may be useful for detection of Ab binding and C3 deposition but may be too elevated for CDC detection. The lack of CDC detection may be due to an excess of infected cells compared to complement. Indeed, this high infection rate is not physiologically relevant, in vivo, the percentage of infected cells is much lower.

Line 340: "Active complement" should be replace by "trigger complement deposition"

Referee #2:

The authors have responded well to the reviewer comments.

Minor point of criticism is that the abstract states "Mutating the Fc of bNAbs to enhance hexamerization potentiates CDC." This is not surprising at all, there are many other papers that have already described the effect of hexamer promoting mutations in Fc, it should be removed from abstract.

Referee #3:

The authors have made an excellent job of addressing my comments and I recommend the manuscript is accepted. I have a few remaining comments but these are for the authors consideration and don't require another review.

1. In Figure 1B, the control antibody mGO53 doesn't bind to FcgR on the Raji B cells and they are 0% +ve. However, in Figure 3 the same antibody is shown to bind FcgR on T cells. This is surprising.

2. It is stated that "The decrease of the fraction of Gag+ cells was more rapid with ΔVpu than with WT-infected cells (Fig 5D)." This isn't clear to me from the data. To support this statement, I think it would be appropriate to show statistics for the comparison. At present, the deltaVpu and WT results aren't statistically compared and Fig 5D only compares antibody responses within each virus.

2nd Revision - authors' response 12 November 2019

Referee #1:

The manuscript form Dufloo et al. was largely improved in this new version. Most of the concerns have been taken into consideration. These modifications largely increases the comprehension of the field and put forward the novelty of the results compared to previous published data. However, the data showing apparent disappearance of infected cells in the presence of complement are doubtful.

We thank reviewer #1 for his/her positive review. We have taken into account his/her concerns about the data presented in figure 5.

First, what is the rational for enrichment of CD4 T infected cells by magnetic depletion of CD4high non infected cells for the protocol of analysis of disappearance of infected cells?

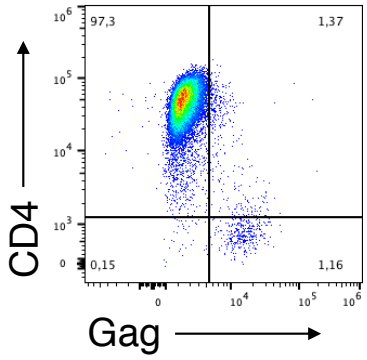
Did author verify that the depletion of CD4+ cells are not infected? It was described that CD4 downregulation was less effective on CD4 primary cells compared to cell lines.

As shown in Figure 2, we did not observe a rapid death of HIV-1-infected cells coated with antibodies and complement. Our next step was then to study the impact of complement deposition over several days. We decided to sort productively infected cells by magnetic depletion of CD4+ cells, in order to eliminate dying cells and to start with a homogenous population of infected cells. We hypothesized that this strategy would help detecting any effect.

This rationale is presented in the text lines 355-358:

"To eliminate dying and bystander cells, we sorted infected cells. Primary CD4 T cells were infected with HIV-1 for one day and the fraction of productively infected cells was enriched by magnetic depletion of non-infected (CD4 high) cells"

In our hands CD4 down-regulation is efficient in HIV-1 infected primary cells, as shown for instance in the flow cytometry plot below. Thus, CD4-based purification is effective to enrich productively infected cells and to remove most of non-infected cells or cells with low levels of viral proteins.



We agree that some infected cells, especially at the early stages of the viral life cycle, may still express high levels of CD4. To avoid any bias, we sorted infected cells before applying any further treatment (antibody and serum). This allowed us to follow the fate of the same population of enriched productively infected cells over time

Moreover, it is known that magnetic beads depletion retain dead cells. This may explain the unexpected low level of cell death cells detected over the 6 days of culture (supplemental figure 5B, 80% living cells). It is also known that unspecific binding via Fc domain of Abs may occur during depletion strategies. These different experimental caveats may differentially impact the percentage of infected cells recovered for the different treatment (NHS, HIHS, with Abs...).

We agree with the reviewer that magnetic separation retains dead cells. This was actually an advantage in our case, since our aim was to follow the fate of living infected cells.

It would be of interest to verify that the protocol of infected cell enrichment used for this manuscript do not interfere with the differential detection of infected cells according to the treatment. Also, please add the detailed protocol for detection of disappearance of infected cells in the Mat and Method section.

As mentioned above, enrichment of infected cells was performed before treatment with serum and antibody. Thus, any difference observed between conditions is not caused by a different processing of the cell during the sorting. We apologize if this was not sufficiently clear in the text.

As suggested by the reviewer, we have modified the methods, which now reads (lines 526-531):

"When indicated, the fraction of live productively infected lymphocytes was enriched by magnetic depletion of non-infected, CD4 high cells present in the population. Depletion was performed after 24h of infection, using LD columns (Miltenyi) and CD4 microbeads (Miltenyi), according to manufacturer instructions. After sorting, up to 90% of the cells were CD4-negative, as controlled by flow cytometry."

And in the text, lines 312-313:

"Enriched WT or ΔVpu NLAD8-infected cells (up to 90% of Gag^+ cells) were then splitted into different cultures and incubated with either 10-1074^{E430G} or isotype control, in the presence of either NHS or HIHS.

Second, the dot plot figure 5 B suggest that cells (number of dots) increased drastically at day 6 in the presence of HIHS in mGO53 and 10-1074 treated cells, but this doesn't seem to be the case in the presence of NHS, with fewer dots, especially when cells are treated with 10-1074. These plots contradict the results of supplementary figure 5 showing continuous increased cell count up to day 6 in presence of NHS or HIHS. Is this cell count corresponding to cells isolated after CD4 infected T cell enrichment?

As observed by the reviewer, there were more events in the conditions containing HIHS compared to NHS. However, there was no clear difference between mGO53 and 10-1074 in the NHS condition (8080 vs 7707 events in the experiment depicted in figure 5B). Thus, NHS may impact growth of infected cells independently of antibodies. We believe that the identification of the underlying mechanisms is beyond the scope of our study, as we focused on antibody-mediated complement activation.

In supplementary figure 5, our aim was to perform a control experiment and to analyze the impact of NHS and HIHS on the growth of non-infected primary CD4 T cells. In figure 5, the objective was to determine how anti-HIV-1 antibodies impact infected cells in the presence of serum.

We modified the legend of figure EV4B to improve clarity. It now reads (lines 925-926):

"Non-infected primary CD4 T cells were incubated or not with 50% NHS or HIHS and cell count and viability were measured each day for 6 days (n=4)."

Third, because of the higher number of cells analyze in HIHS and NHS/GO53 treated cells at day 6, the percentage of gag positive cells may be overestimated compared to NHS/10-1074 treated cells. Indeed the separation between gag+ and gag - is not easy to define, and the gate may need to be heightened. To finely tune the gag positive cells, controls without gag staining should be shown. It would also be of interest to show plot with lower number of cells analyzed similarly as 10-1074 treated cells to better define the gates.

With respectfully disagree with the reviewer that a modification in the number of events may change the frequency. Indeed, if we detect 10 infected out of 100 total cells, or 100 infected out of 1000 total cells, we will each time measure 10% of infected cells.
To improve clarity, we have reduced the size of the dots in figure 5. This allows a better view of the number of events and improves the separation between Gag^+ and Gag^- cells.

Fourth, the disappearance of infected cells with WT virus is not at all obvious at day 1, 2 and 3 (supplemental figure 5A). With delta-Vpu infected cells, the % of gag+ cells is lower only for one point below 0.1 corresponding to 1 donor day 3. It would have been of interest to label each of the n=6 experiments to identify the corresponding dots for each separate experiment. To compare the role of complement in WT and delta-Vpu infected cells, the % of infected cells recorded with these two viruses should be mentioned.

We agree with reviewer #1 that antibody and complement-dependent disappearance of WT-infected cells is not obvious at day 1, 2 and 3. This actually confirms results from figure 2, which shows that there is no CDC at 24h, and further extend this finding to days 2 and 3. The strongest differences are observed at day 6, which is presented in figure 5C.

Concerning ΔVpu, at day 3, every donor displays a reduction between NHS and HIHS when treated with 10-1074. To improve clarity, we have labelled each donor in Figure EV4 and Figure 5.

The % of infected cells recorded for WT and ΔVpu-infected viruses are depicted in Figure EV4 (days 1, 2 and 3) and Figure 5 (day 6).

Fifth, a decreased % of gag expressing cells is systematically detected in cells treated with mGO53 control Ab in the presence of NHS compared to HIHS. This suggest that NHS has a direct inhibitory effect on infected cells. An additional control without Ab should be added to estimate the relative contribution of mGO53 Ab compared to complement alone. Does this inhibition means that complement has a direct effect on infected cell disappearance? Is this inhibition due to additional factors present in the NHS that is inactivated in HIHS?

We agree with the reviewer that an inhibitory effect of the NHS by itself is visible, as already stated in line 310-312:

"The frequency of HIV-1 infected cells was lower in cultures containing NHS, as compared to HIHS, suggesting that the complement by itself may impact the survival of infected cells".

However, we believe that studying this effect is beyond the scope of our report, which focuses on complement activation by antibodies.

Additional comments:

EV1A: The % of Gag infected cells seems extremely and unusually high for primary CD4 T cell infection with primary virus, even higher as in figure 5B where cells were infected with AD8 and enriched. Is this high infection due to DEAE/Dextran treatment? Did authors enriched infected cells by CD4 high magnetic depletion? This high rate of infected cells may be useful for detection of Ab binding and C3 deposition but may be too elevated for CDC detection. The lack of CDC detection may be due to an excess of infected cells compared to complement. Indeed, this high infection rate is not physiologically relevant, in vivo, the percentage of infected cells is much lower.

The % indicated in the gates correspond to the frequency of ENV-positive cells among infected cells, not to the frequency of infected cells among the total population. In figure 6 (previously EV1) the frequency of infected cells is 27%, 30% and 6% (for CH058, CH077 and vKB18, respectively), which is not uncommon in our conditions with primary CD4 T cells.

To avoid any confusion, we have modified the legend, which now states (lines 890-891):
"The percentages of infected cells were 27%, 30% and 6% for CH058, CH077 and vKB18, respectively."

The lack of CDC is not due to the frequency of infected cells, as the same conditions allowed 60-90% of CDC with W6/32 antibody (targeting HLA-A/B/C), demonstrating that the lack of CDC is not due to an excess of target.

Line 340: "Active complement" should be replace by "trigger complement deposition"

The sentence has been modified.

Referee #2:

The authors have responded well to the reviewer comments.

We thank reviewer #2 for taking the time to assess our work and for his/her positive review.

Minor point of criticism is that the abstract states "Mutating the Fc of bNAbs to enhance hexamerization potentiates CDC." This is not surprising at all, there are many other papers that have already described the effect of hexamer promoting mutations in Fc, it should be removed from

abstract.

This sentence has been removed from the abstract.

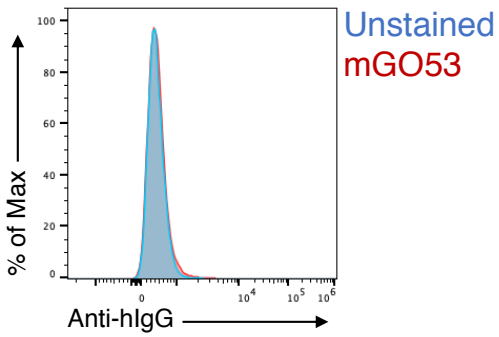
Referee #3:

The authors have made an excellent job of addressing my comments and I recommend the manuscript is accepted. I have a few remaining comments but these are for the authors consideration and don't require another review.

We thank reviewer #3 for his/her positive and critical reading of our work.

1. In Figure 1B, the control antibody mGO53 doesn't bind to FcgR on the Raji B cells and they are 0% +ve. However, in Figure 3 the same antibody is shown to bind FcgR on T cells. This is surprising.

mGO53 does not bind to CD4 T cells. This was not clear from the figure, as we did not include the unstained control. As shown below, the MFI is identical between mGO53 and an unstained control. This is expected as CD4 T cells do not usually express FcR.



We have added the following sentence in the legend (lines 775-776): "The staining obtained with mGO53 was similar to the background signal observed on unstained cells"

2. It is stated that "The decrease of the fraction of Gag+ cells was more rapid with ΔVpu than with WT-infected cells (Fig 5D)." This isn't clear to me from the data. To support this statement, I think it would be appropriate to show statistics for the comparison. At present, the deltaVpu and WT results aren't statistically compared and Fig 5D only compares antibody responses within each virus.

We have performed a statistical analysis and included it in the text, which now reads (lines 316-319):

"At day 3, the relative percentage of Gag+ cells with 10-1074 in NHS compared to HIHS, was lower with ΔVpu than with WT-infected cells (58% and 26% for WT and ΔVpu, respectively; p=0.0312; Wilcoxon test) (Fig 5D)."

Accepted 15 November 2019

Thanks for the submission of the final revised version of your manuscript. I now went through your point-by-point response, and I consider the remaining concerns of the referees as adequately addressed. Thus,
I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Olivier Schwartz and Timothée Bruel
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2019-49351-T

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	yes, see figure legends and methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA. Only non-parametric test were used

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Is there an estimate of variation within each group of data?	error bar or individual values are used in each figure.
Is the variance similar between the groups that are being statistically compared?	NA. Only non-parametric test were used

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	yes. See methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells were obtained from ATCC and tested for mycoplasma contamination. See methods

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Study was approved by the "Comité de Protection des Personnes" (Ile-de-France VII, Paris, France). This is indicated in the methods.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	The study was performed according to European guidelines and the Declaration of Helsinki. Each participant provided written consent to participate. These are indicated in the methods.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA