nature portfolio

Peer Review File

Long-term cellular immunity of vaccines for Zaire Ebola Virus Diseases

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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Comments to the authors

The authors investigated the long-term function, breadth, and magnitude of vaccine-elicited memory T-cell responses for the Ad26-MVA, rVSV, and rVSVbooster vaccines. Thirty-one participants were randomly selected from the PREVAC immunological ancillary study which enrolled 196 (191 per-protocol) adult participants from the PREVAC trial, to assess long term cellular immune responses. I think the manuscript is well written and addresses an important question, however I believe there are some points that should be clarified prior to publication.

General comments

1. By the title and description of the manuscript it seems like the goal is to focus on the long-term immunity of these vaccines. However, as I read the manuscript it seems like a large portion of the results (including figures) are focused on presenting some of the short-term outcomes which in some cases appear to be mostly confirmatory. This is fine, but I think then this should also be more clearly listed as an objective of this work.
2. As I was reading the manuscript at first, I thought the entire population of the immunological study was included, but then realized that in some cases all 191 were included, in others a subset 92 participants, in others a subset of 31. Can the authors explain why these subgroups were selected, and how they were chosen from the 191 participants? The treatment allocation was randomly assigned but were these subsets stratified random samples of the 191 participants or was it a convenience sample?

Statistical comments:

1. Please provide more details about the bivariate model used for comparison between active and placebo groups. I appreciate the authors presented a citation for the model, but a general description (at the least) should be available for the reader in the manuscript.
2. Please provide more details about how the multiple comparison procedure was implemented. I appreciate the authors presented a citation for the model, but a general description (at the least) should be available for the reader in the manuscript. There are several ways in which a multiple comparison procedure can be implemented, for example looking at figure 1 looking at IL-18, at each visit there are 3 possible comparisons: gray to red, gray to blue, red to blue, was the procedure implemented for those 3 comparisons, or for all 9 considering the three visit days, or including all the comparisons at all visit days for all cytokines? The corrections would be different, going from the least conservative (correcting by visit) to the most conservative (correcting at all visits for all cytokines).
3. In the correlations analysis, it looks like it was stated that the EBOV-specific CD4+ T-cell responses were positively correlated with the antibody titers from D12 to M12. From the graph it appears that several of these correlations while significantly different from zero are quite small in magnitude, particularly at M12. Is the interest here to only demonstrate a correlation and the magnitude is not as important for this work, or should there be a clarification on that in the text?

Reviewer #2:
Remarks to the Author:
General:

1. This paper uses participants who were vaccinated during the PREVAC study, presents some antibody data that was collected during the PREVAC study, and performs fresh analyses on blood samples collected during and long after the PREVAC study. Because of the intertwined nature of this paper's efforts with a previous study, it is recommended the authors generate a figure illustrating the timeline of the studies and sample collections.

2. Line 99: Why were the participants randomized into these uneven groups? Did this randomization occur in the initial PREVAC study and these are numbers of each group that are currently participating in the follow-up? Are these data points representing individuals who participated in the full follow-up study, were new people sampled to address each timepoint, or do these numbers only represent those who participated for the full duration of the follow-up? If so, were there data points representing people who stopped participating partway through the follow up and then were excluded? This is not clearly discussed in the methods section.

3. Methods: Trial design and participants: This section makes extensive references to different protocol versions, as well as a "main study", and then states that those patients who underwent version 3.0 were excluded due to variation. This results in approximately 10 lines of text which only confuse those readers not already familiar with the evolution of these studies. It is recommended to clearly state, or use a figure to illustrate, those vaccine regimens that participants did receive, and then simply state that participants who received alternate vaccine regimens were excluded from this follow-up study. Additionally, explain the dosages, injection strategies, and other details involved in those vaccinations.

4. Methods: Line 346: "Samples from a subset of the immunological ancillary study participants randomized in active vaccine arms in PREVAC version 4.0 were randomly selected for assessment . . ." How many samples were selected per cohort per analysis? These numbers should be stated in both the methods and results.

5. As the primary concern is the impact of the different viral vectors upon immune response, please include a paragraph in the introduction or discussion explaining the expected differences in the immune responses promoted by these vectors based upon the literature.

6. Some emphasis is placed upon CD4 T cells and G-CSF in the discussion. What conclusions do the reviewers draw from this and other examined markers regarding potential immune pathway bias and/or implications for increased durability of humoral responses between the different vaccine regimens?

7. The authors write: "Our findings could guide booster vaccination recommendations and help identify populations likely to benefit from revaccination." in the Summary, a claim later repeated. This reviewer fails to see where in the manuscript the authors link data to support new guidance on the need to boosting, when and with what vaccine and/or identify population likely to benefit (most) from a booster dose. In the purely speculative world, the author's above statement is possible if not likely. Then again everything is in that speculative world. Could the authors better link data to support this concept? If not, this may be aspiration better positioned in the Discussion labeled as speculative or hypothetical at the minimum.

Specific Comments

1. As humoral immunity appears to have been the primary focus of previous studies and is used as a correlate of protection, is there an antibody titer that is currently used as a threshold for protective immunity?

2. Line 87: "As the current vaccines rely on viral vector platforms, there may be differences in terms

of durability of such responses." Do you specifically mean that because one construct is adenovirus and one is VSV there may be a difference based upon the viral vectors used?

3. Throughout the paper, clearly label which immunizations and timepoints relate to the initial PREVAC study, and which are occurring as part of the current follow-up study. For example, the paragraph starting at line 341 is unclear in this regard.

4. Line 172: "we detected no significant levels of specific CD8+T cells in the rVSV-booster group" Is this correct? In figure 5 it looks like the M48 timepoint is labelled as significantly different.

5. Line 291-293: Elaborate with specifics on these disparities.

6. Figure 3A should state which cytokine signals are being examined in the graph.

7. Figure 5: make the differences between non-stimulated cells and EBOV stimulated cells more distinct.

8. Figure 7: Recommend adding borders or other lines to distinguish between vaccine groups being compared.

Reviewer #3:
Remarks to the Author:
Overall, a worthwhile study that addresses the durability and polyfunctionality of T cell responses in humans after receiving different approved viruses vectored Ebolavirus vaccines. This is an area that is neglected in comparison to antibody responses. The manuscript is well written and adds to our understanding of the durability of the vectored vaccine response, with the T cell polyfunctionality being the most novel aspect. The authors should be commended for reducing highly multidimensional data into impactful Figures.

A major conclusion from the study is there are differences between the vaccines' ability to induce polyfunctional CD4 T cells. Polyfunctionality increases significantly after Ad26 prime (Fig. 3A D14), which is improved by MVA boost (Fig 3A D70), and in the rVSV booster group at D70 (Fig. 3A D70). The authors state this was not seen in CD8 cells or in the rVSV prime only group, or by M12. However, the pie charts to support these statements (panel B) include only the significant samples, while the non-significant CD8, rVSV prime only and M12 plots are omitted.

My expectation would be that there would be a larger proportion of cells producing low numbers of cytokines in the non-significant groups, but it is frustrating not to be able to see this in the pie charts. These omitted pie charts need to be included to support the conclusions; if they don't support the conclusions this needs to be addressed.

Also, the study seems to avoid performing statistical comparisons between the vaccines, only comparisons within each vaccine group between placebo and vaccine at different time points. Such tests would help assess the relative performance of the vaccines.

Minor comments
Figure 1. Profiling on D7 detected elevation of only 3 out of 67 markers measured. Sampling on D7 will likely miss much of the inflammatory response seen in the first few hours. Please provide a

rationale for this time point.
Fig. 2. No P values between vaccines, or vaccines and placebo are given. Please add some stats.
Fig. 3A. Line 136 states “There were no detectable specific T-cell produced cytokines after a single dose of rVSV”. On D70 there seem to be positives but this fails to reach significance, and yet the rVSV booster group, which looks very low, reaches significance. This is difficult to believe looking at the box plots. The Figure might benefit from adding the % positives mentioned in the narrative over each column.
Fig. 3A. Why is the rVSV prime only group lacking from D14 plots?
Fig. 3A. The placebo group seems very high in the CD4 M12 plot. If this is an illusion because the N is greater but most are at baseline, again I recommend the % positive values are placed over each column.
Fig. 4. Wy were the data for different vaccines pooled? If the vaccines are analyzed separately, do any stand out as different?
Fig. 5. rVSV boosting induced EBOV-specific CD4+ T-cell responses but failed to stimulate EBOV-specific CD8+ T-cell, whereas the single-dose regimen induced both. The authors suggest pre-existing immunity in the booster group blunts the recall response; can the authors speculate why this is confined to CD8 cells?
Fig 5 (and other Figs). P-values: what is the rationale for NOT comparing between vaccines, only between placebo and different time points within each vaccine group.
Fig. 7. The legend is unreadable. Please enlarge.
Fig. 7. Given Th2 cytokines were detected in the supernatants of peptide-restimulated PBMC, could the authors explain why they didn’t perform a multifunctionality study like Fig. 3 for Th2 cells.

Authors' responses

- Reviewer #1 (Remarks to the Author):

Comments to the authors

The authors investigated the long-term function, breadth, and magnitude of vaccine-elicited memory T-cell responses for the Ad26-MVA, rVSV, and rVSVbooster vaccines. Thirty-one participants were randomly selected from the PREVAC immunological ancillary study which enrolled 196 (191 per-protocol) adult participants from the PREVAC trial, to assess long term cellular immune responses. I think the manuscript is well written and addresses an important question, however I believe there are some points that should be clarified prior to publication.

General comments

1. By the title and description of the manuscript it seems like the goal is to focus on the long-term immunity of these vaccines. However, as I read the manuscript it seems like a large portion of the results (including figures) are focused on presenting some of the short-term outcomes which in some cases appear to be mostly confirmatory. This is fine, but I think then this should also be more clearly listed as an objective of this work.

Authors' response: We thank the reviewer for this comment and asking us to clarify. We have studied T-cell responses to vaccination both in the short and long term after vaccine injection. We have modified the text in the introduction accordingly (page 4 lines 91-93). We believe one of the strengths of our study is the long-term follow-up of participants in Guinea, despite the logistical challenges involved.

2. As I was reading the manuscript at first, I thought the entire population of the immunological study was included, but then realized that in some cases all 191 were included, in others a subset 92 participants, in others a subset of 31. Can the authors explain why these subgroups were selected, and how they were chosen from the 191 participants? The treatment allocation was randomly assigned but were these subsets stratified random samples of the 191 participants or was it a convenience sample?

Authors' response: We acknowledge that this point needs further clarification.

Participants included in this PREVAC immunological ancillary study (n=230 in total) were originally a subset of adult participants at the Landreah site (Guinea) randomized to one of the three vaccine strategies or to a placebo group as part of the PREVAC main trial. Participants from different PREVAC protocol versions V2.0, V3.0 and V4.0 were pooled for this ancillary study, except for those who received a diluted rVSVΔG-ZEBOV-GP vaccine in V3.0 as this vaccine strategy was only evaluated for safety reasons before moving on to the final licensed dose (undiluted dose) in PREVAC V4.0. Consequently, T cell analyses until month 12 were performed on 196 participants, of which 191 participants were included in the per-protocol population after exclusion of five participants (HIV-positive test (n=4) or discontinuation of the vaccine protocol (n=1)).

Serum concentrations of IgG binding antibodies against the Ebola virus surface glycoprotein were measured at baseline and at each follow-up visit until M12 only for participants recruited in the version 4.0 of PREVAC. Thus, only a subset of 92 among the 191 participants had these IgG measurements.

For the long-term follow-up at M24, M36, M48 and M60, samples from a subset of the immunological ancillary study participants randomized in active vaccine arms in PREVAC version 4.0 were randomly selected for the assessment of long-term responses for feasibility

reasons. Since there were only 8 patients in the rVSV-booster arm of the ancillary study, we conducted those experiments on a similar number of patients from the other vaccine arms (11 in the Ad26/MVA group and 12 in the rVSV group) to minimize imbalance between the groups. Finally, those experiments were performed in n=31 participants. We have clarified this point in the Methods section of the manuscript (p15-16).

Statistical comments:

Please provide more details about the bivariate model used for comparison between active and placebo groups. I appreciate the authors presented a citation for the model, but a general description (at the least) should be available for the reader in the manuscript.

Authors' response: Thank you for your comment, we have added more details on this model developed by our team specifically for analyzing this type of data and published in J Immunol Methods in 2020 (see Methods, page 18/19 lines 424-427). This modeling approach showed good statistical performances for measuring vaccine effect whatever the relationship between non-stimulated and stimulated responses:

"The bivariate model was built to analyze the T-cell responses measured by ICS in vaccine trials taking into account unstimulated control response and stimulated response by antigens, irrespective of the correlation between the non-specific and specific responses"

Reference: Lhomme E, Hejblum BP, Lacabaratz C, Wiedemann A, Lelièvre JD, Levy Y, Thiébaut R, Richert L. Analyzing cellular immunogenicity in vaccine clinical trials: a new statistical method including non-specific responses for accurate estimation of vaccine effect. J Immunol Methods. 2020 Feb;477:112711. doi: 10.1016/j.jim.2019.112711. Epub 2019 Dec 3. PMID: 31809708.

2. Please provide more details about how the multiple comparison procedure was implemented. I appreciate the authors presented a citation for the model, but a general description (at the least) should be available for the reader in the manuscript. There are several ways in which a multiple comparison procedure can be implemented, for example looking at figure 1 looking at IL-18, at each visit there are 3 possible comparisons: gray to red, gray to blue, red to blue, was the procedure implemented for those 3 comparisons, or for all 9 considering the three visit days, or including all the comparisons at all visit days for all cytokines? The corrections would be different, going from the least conservative (correcting by visit) to the most conservative (correcting at all visits for all cytokines).

Authors' response: We agree with the reviewer's comment. We used the Benjamini-Hochberg method, in which we adjusted for each arm separately, but across visits and across markers for each type of analysis (Luminex, ICS). We have clarified this point in the method section of the manuscript (see page 14 lines 434-437):

"We used a FDR method (Benjamini-Hochberg method) to adjust for test multiplicity for dependent comparisons (adjustment for each arm separately, across visits and across markers for each type of analysis)."

3. In the correlations analysis, it looks like it was stated that the EBOV-specific CD4+ T-cell responses were positively correlated with the antibody titers from D12 to M12. From the graph it appears that several of these correlations while significantly different from zero are quite small in magnitude, particularly at M12. Is the interest here to only demonstrate a correlation and the magnitude is not as important for this work, or should there be a clarification on that in the text?

Authors' response: We agree with the reviewer that although the observed Spearman correlation matrix showed that EBOV-specific CD4+ T-cell responses detected at D14 were positively correlated with antibody titers from D14 to M12, these correlations remain weak to moderate. We have clarified this in the results (Results, page 7 lines 153-154):

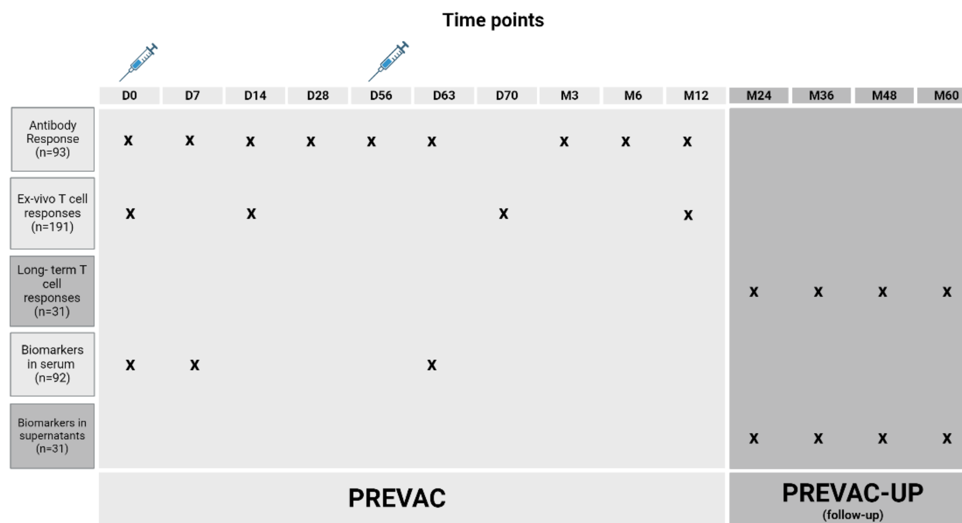
"We then estimated the correlations between antibody responses from D0 to M12 and EBOV-specific ex-vivo CD4+ T-cell responses from D14 to M12 in all vaccine groups. The Spearman correlation matrix showed that the EBOV-specific CD4+ T-cell responses detected on D14 positively correlated with the antibody titers from D14 to M12, with weak to intermediate correlation coefficients."

- Reviewer #2 (Remarks to the Author):

General:

1. This paper uses participants who were vaccinated during the PREVAC study, presents some antibody data that was collected during the PREVAC study, and performs fresh analyses on blood samples collected during and long after the PREVAC study. Because of the intertwined nature of this paper's efforts with a previous study, it is recommended the authors generate a figure illustrating the timeline of the studies and sample collections.

Authors' response: We appreciate the reviewer's suggestion and have revised Supplementary Figure 2 to clarify the timelines and better distinguish between the PREVAC study and the PREVAC follow-up study. Please refer to the updated figure below.



2. Line 99: Why were the participants randomized into these uneven groups? Did this randomization occur in the initial PREVAC study and these are numbers of each group that are currently participating in the follow-up? Are these data points representing individuals who participated in the full follow-up study, were new people sampled to address each timepoint, or do these numbers only represent those who participated for the full duration of the follow-up? If so, were there data points representing people who stopped participating partway through the follow up and then were excluded? This is not clearly discussed in the methods section.

Authors' response: The participants were randomized to one of the three vaccine strategies described (page 5 lines 101-102)or to a placebo group as part of the PREVAC main trial. The allocation of participants to groups follows the PREVAC randomization ratio (2:1:2:1:1). However, the distribution also reflects the fact that PREVAC version 2.0 included only Ad26/MVA as the active vaccine arm, and participants receiving rVSV were only included in version 4.0 of PREVAC. Participants from all protocol versions (V2.0, V3.0 and V4.0) were pooled for this ancillary study, except for those who received a diluted rVSVΔG-ZEBOV-GP vaccine in V3.0 as this vaccine strategy was only evaluated for safety reasons before moving on to the final licensed dose in PREVAC V4.0.

All participants included in the sub-study followed the protocol of the main study and the study follow-up. As specified at the beginning of the results (page 5 line 99), only 5 participants were excluded of the per-protocol population due to an HIV-positive test (n=4) or discontinuation of the vaccine protocol (n=1).

3. Methods: Trial design and participants: This section makes extensive references to different protocol versions, as well as a "main study", and then states that those patients who underwent version 3.0 were excluded due to variation. This results in approximately 10 lines of text which only confuse those readers not already familiar with the evolution of these studies. It is recommended to clearly state, or use a figure to illustrate, those vaccine regimens that participants did receive, and then simply state that participants who received alternate vaccine regimens were excluded from this follow-up study. Additionally, explain the dosages, injection strategies, and other details involved in those vaccinations.

Authors' response: We would like to thank the referee to help us to clarify the presentation of this complex study. The complexity arises from the three different versions of the PREVAC protocol, under which the PREVAC ancillary study participants were included. We have revised this section in the Method section for greater clarity. Dosages and injection strategies have been added.

4. Methods: Line 346: "Samples from a subset of the immunological ancillary study participants randomized in active vaccine arms in PREVAC version 4.0 were randomly selected for assessment . . ." How many samples were selected per cohort per analysis? These numbers should be stated in both the methods and results.

Authors' response: As mentioned lines 104-107 (Results section), "a *total of 31 randomly selected individuals - 11 (35.5%) from the Ad26-MVA, 12 (38.7%) from the rVSV, and 8 (25.8%) from the rVSV-booster groups – were used to assess long term cellular immune responses*". The socio demographic characteristic of these participants are described in Supplementary Table 1. All these participants were from the three active arms of PREVAC version 4.0. We have added precision in the Methods (page 16 lines 355-358):

"*Then, samples from a subset of 31 study participants randomized in active vaccine arms in PREVAC version 4.0 (11 from the Ad26-MVA, 12 from the rVSV, and 8 from the rVSV-booster groups) were randomly selected for the assessment of long-term responses at 24 (± 6 months), 36 (± 6 months), 48 (± 6 months), and 60 (− 6 months; + 1 month) months within the PREVAC-UP trial.*"

5. As the primary concern is the impact of the different viral vectors upon immune response, please include a paragraph in the introduction or discussion explaining the expected differences in the immune responses promoted by these vectors based upon the literature.

Authors' response: We appreciate the reviewer's suggestion. We have outlined the distinctions in immune responses induced by Ad26/MVA and rVSV in the introduction (lines 55-70). Furthermore, we have expanded upon this description in the discussion section of the revised manuscript (lines 293-301).

6. Some emphasis is placed upon CD4 T cells and G-CSF in the discussion. What conclusions do the reviewers draw from this and other examined markers regarding potential immune pathway bias and/or implications for increased durability of humoral responses between the different vaccine regimens?

Authors' response: We do agree with the referee that the interpretation of factor dosages in the supernatant of stimulated PBMCs from vaccinated individuals should be approached with caution. Although we cannot entirely rule out some biases in these observations (such as non-specific cell activation and donor heterogeneity), we believe that these observations are significant. Thanks to the longitudinal analyses, these findings are consistent across different time points and vaccine arms for some of these factors. As stated lines 203-206, "*At M36, we observed the presence of the same cytokines as previously identified in both study arms. By contrast, at M48 and M60, only Th1 cytokines (IFN-g, IL-2) and G-CSF were still observed in the Ad26-MVA arm. Only G-CSF and the pro-inflammatory cytokine IL-1b were detected at M60 in the rVSV group (Figure 7)*". Regarding G-CSF, we tempered the possible interpretations by indicating that this factor primarily reflects T-cell activation.

7. The authors write: "Our findings could guide booster vaccination recommendations and help identify populations likely to benefit from revaccination." in the Summary, a claim later repeated. This reviewer fails to see where in the manuscript the authors link data to support new guidance on the need to boosting, when and with what vaccine and/or identify population likely to benefit (most) from a booster dose. In the purely speculative world, the author's above statement is possible if not likely. Then again everything is in that speculative world. Could the authors better link data to support this concept? If not, this may be aspiration better positioned in the Discussion labeled as speculative or hypothetical at the minimum.

Authors' response: We appreciate the reviewer's comment. While we do agree that the necessity of a booster remains speculative, this option is currently under discussion within the PREVAC consortium in collaboration with J&J and Merck. A trial to test the booster effect of both the Ad26/MVA and rVSV vaccines is under discussion in the Democratic Republic of the Congo (EBO-BOOST trial, NCT06126822).

We believe that we addressed this option cautiously in the discussion (lines 278-281): "*In the same vein, the lack of data on the durability of vaccine efficacy hampers a clear definition of the criteria for re-vaccination. To date, there are no immunological indications for a booster vaccination or indications concerning the population that would likely benefit*". In the discussion (lines 281-285), we attempted to propose a link between our data and the potential need for a booster: "*As CD4+ T cells play a pivotal role in promoting the development and persistence of humoral responses, our demonstration of a correlation between EBOV-specific T-cell responses and anti-EBOV IgG responses may provide a rationale for the need of a long-term boost to maintain T- and B-cell memory responses.*"

Regarding the population likely to benefit from a booster: Although our data revealed the long-term persistence of memory T cells after in vitro stimulation assay, ex vivo analysis of T-cell responses showed different profiles depending on the vaccine regimen (Ad26/MVA, rVSV, rVSV-booster). The lack of ex vivo T-cell responses in the rVSV group (one shot) compared to

the Ad26/MVA or rVSV-booster groups may indicate that individuals vaccinated with a single rVSV dose (which is the current recommendation) might benefit from a booster dose.

Specific Comments

1. As humoral immunity appears to have been the primary focus of previous studies and is used as a correlate of protection, is there an antibody titer that is currently used as a threshold for protective immunity?

Authors' response: This is an important question that remains unresolved. Identifying Ebola correlates of protection in humans is challenging because they can only be analyzed during outbreaks, where the emergency situation limits the planning and execution of clinical studies. Correlates of protection may differ between infection-induced and immunization-induced immunity. Although antibodies are believed to play a crucial role in vaccine-mediated protection against Ebola virus, specific immune correlates of protection against the disease have yet to be identified.

As demonstrated in our previous study (Wiedemann et al., Nature Communications, 2019) and other studies, both humoral and cellular immunity are present in EVD survivors. However, their relative contributions to protection in humans remain unknown. [N.J. Sullivan, J.E. Martin, B.S. Graham, G.J. Nabel, Correlates of protective immunity for Ebola vaccines: implications for regulatory approval by the animal rule, Nat. Rev. Microbiol. 7 (2009) 393–400].

The efficacy of a single intramuscular dose of rVSVΔG-ZEBOV-GP (2×10^7 PFU) was demonstrated in an open-label, cluster-randomized ring vaccination trial conducted during the latter part of the 2014 Ebola outbreak in Conakry, Guinea, and Sierra Leone. The study included 11,841 participants, organized into 117 clusters (rings), who were vaccinated either immediately or 21 days after known contact with an EVD case. No EVD cases occurred among vaccinated individuals from day 10 after vaccination, indicating high vaccine efficacy in a peri-exposure context (Henao-Restrepo, A.M., et al. The Lancet 389, 505-518 (2017).

As stated in our manuscript discussion, comparing vaccine-induced immune responses to the natural immune response from infection could help identify correlates of protection against EVD.

2. Line 87: "As the current vaccines rely on viral vector platforms, there may be differences in terms of durability of such responses." Do you specifically mean that because one construct is adenovirus and one is VSV there may be a difference based upon the viral vectors used?

Authors' response: Previous reports have demonstrated differences in immune responses induced by the Ad26/MVA and rVSV vaccines. The Ad26/MVA strategy has been shown to induce robust humoral and cellular immune responses in European and African populations, with CD8 T cells playing a major role. In contrast, vaccination with a single dose of rVSV induces EBOV GP-specific IgG antibody responses in almost all participants *(S.B. Kennedy, N. Engl. J. Med. 377 (2017); A. Huttner, Lancet Infect. Dis. 15 (2016); B.-A.G. Coller, Vaccine 35 (2017))*, while the magnitude of rVSV-induced T cell responses is very low, as we showed in our study and as previously reported (Raabe, V., et al. Vaccine 41 (2023)). The difference in vaccine immunogenicity - particularly T cell responses, could have a major impact on the durability of humoral responses. T cell responses are crucial for the production of antibodies by B cells. Therefore, differences between vaccines in the maintenance of T cell responses could significantly impact the long-term persistence of antibody levels. The results of antibody responses in the follow-up study PREVAC-UP (EDCTP2 project ongoing) will be crucial in addressing this question.

3. Throughout the paper, clearly label which immunizations and timepoints relate to the initial PREVAC study, and which are occurring as part of the current follow-up study. For example, the paragraph starting at line 341 is unclear in this regard.

Authors' response: To elucidate this point, we have revised Supplementary Figure 2, which depicts the sampling schedule and immunological assays, to clearly differentiate between the initial PREVAC study and the current follow-up study (PREVAC-UP). Additionally, we have clarified this distinction within the manuscript.

4. Line 172: "we detected no significant levels of specific CD8+T cells in the rVSV-booster group" Is this correct? In figure 5 it looks like the M48 timepoint is labelled as significantly different.

Authors' response: We agree with the reviewer and have added a sentence in the revised manuscript (please refer to line 174)

5. Line 291-293: Elaborate with specifics on these disparities.

Authors' response: We have incorporated the reviewer's suggestion by adding this paragraph to the discussion section page 13 lines 294-302 (see below)

"However, studies have shown substantial immunological disparities between vaccination and natural Ebola virus infection. In EVD survivors, both humoral and cellular immunity are present, but their specific roles in protection are unclear [37]. Vaccination with VSV- or adenovirus-vectored vaccines demonstrated that both humoral and cellular immunity contributed to protection in NHP[38]. Vaccination with recombinant serotype 5 adenovirus encoding Ebolavirus GP induced anti-GP specific antibodies, but these antibodies alone did not confer protection, as the transfer of EBOV GP-specific IgG from Ad5-EBOV vaccinated NHPs to naïve animals did not protect them against death following EBOV challenge. However, when CD8+ T cells were depleted, 4 out of 5 vaccinated animals died after challenge, indicating a crucial role for these cells [31]. *These disparities provide compelling evidence that antibody titers alone may not suffice for evaluating the antibody-mediated immunity elicited by vaccination[37]"*

6. Figure 3A should state which cytokine signals are being examined in the graph.

Authors' response: As indicated in the legend of Figure 3, the percentage of total cytokines (IFN-γ, IL-2, MIP1β, and TNF) is depicted. This information is now explicitly stated on the y-axis of Figures 3 and 4.

7. Figure 5: make the differences between non-stimulated cells and EBOV stimulated cells more distinct.

Authors' response: We thank the reviewer for the suggestion. We have changed the color, and the unstimulated condition is now represented in grey.

8. Figure 7: Recommend adding borders or other lines to distinguish between vaccine groups being compared.

Authors' response: We thank the reviewer for the suggestion. We have added dashed lines and increased the font size of the legend. We have modified the figure to make it more understandable.

- Reviewer #3 (Remarks to the Author):

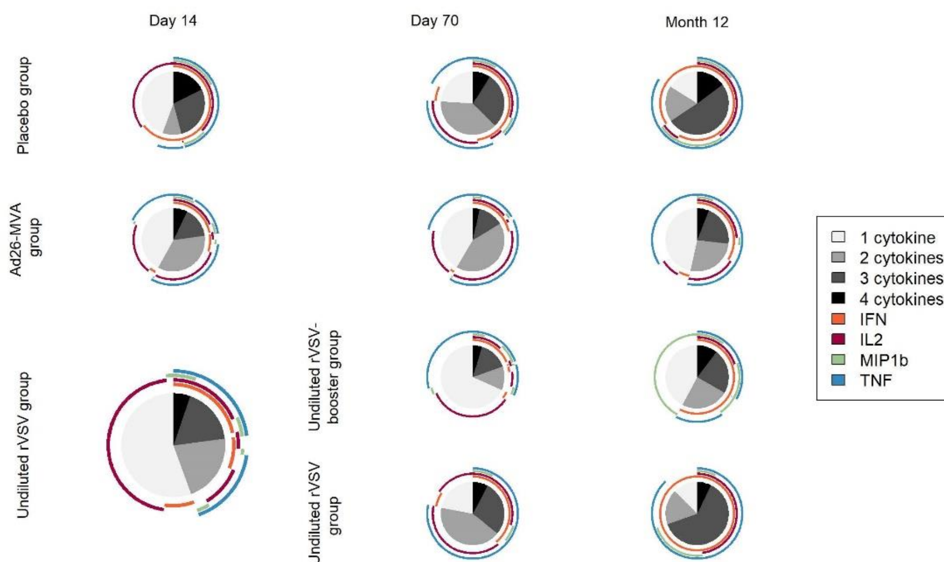
Overall, a worthwhile study that addresses the durability and polyfunctionality of T cell responses in humans after receiving different approved viruses vectored Ebolavirus vaccines. This is an area that is neglected in comparison to antibody responses. The manuscript is well written and adds to our understanding of the durability of the vectored vaccine response, with the T cell polyfunctionality being the most novel aspect. The authors should be commended for reducing highly multidimensional data into impactful Figures.

A major conclusion from the study is there are differences between the vaccines' ability to induce polyfunctional CD4 T cells. Polyfunctionality increases significantly after Ad26 prime (Fig. 3A D14), which is improved by MVA boost (Fig 3A D70), and in the rVSV booster group at D70 (Fig. 3A D70). The authors state this was not seen in CD8 cells or in the rVSV prime only group, or by M12. However, the pie charts to support these statements (panel B) include only the significant samples, while the non-significant CD8, rVSV prime only and M12 plots are omitted. My expectation would be that there would be a larger proportion of cells producing low numbers of cytokines in the non-significant groups, but it is frustrating not to be able to see this in the pie charts. These omitted pie charts need to be included to support the conclusions; if they don't support the conclusions this needs to be addressed.

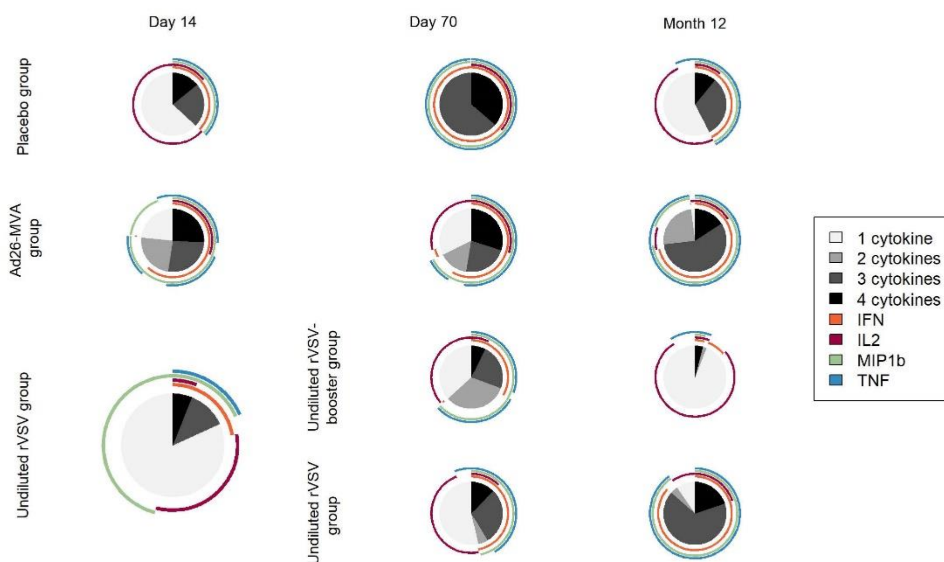
Authors' response: A major conclusion of the study is that there are differences in the vaccines' ability to induce EBOV-specific CD4 T cells. Using Ex vivo ICS, we demonstrated that one injection of Ad26 induced specific CD4 T cells, and this response was boosted by the MVA injection. For the rVSV vaccine, two injections were necessary to detect ex vivo EBOV-specific CD4 T cells (see Figure 3).We then analyzed the polyfunctionality of the EBOV-specific CD4 T cells using Boolean gates to determine the functionality of these specific CD4 T cells. Evaluating polyfunctionality is important, as it has been shown that polyfunctional T cells play a major role in vaccine-induced immune responses. Consequently, polyfunctionality is only assessed when specific T cells are detectable. Moreover, the results of polyfunctionality are presented with background subtraction. Based on this, it is uncommon to represent the polyfunctionality of non-specific cells, as this would essentially be examining the polyfunctionality of the background.

However, to better address the reviewer's comment, the figure below shows pie chart of all cells including "non-responder cells," which is not different from the background and therefore not considered "specific responses".

- **Polyfunctionality of CD4 T cells:**



- **Polyfunctionality of CD8 T cells:**



Also, the study seems to avoid performing statistical comparisons between the vaccines, only comparisons within each vaccine group between placebo and vaccine at different time points. Such tests would help assess the relative performance of the vaccines.

Authors' response: We thank the reviewer for this comment. The PREVAC trial, was methodologically designed to compare each active vaccine strategy to the placebo arm, respectively (and not the active vaccine strategies amongst each other). The trial is not powered to compare the different vaccines directly. It was also an agreement between the various consortium members, including industrial partners, not to compare active vaccines directly with each other.

Minor comments

Figure 1. Profiling on D7 detected elevation of only 3 out of 67 markers measured. Sampling on D7 will likely miss much of the inflammatory response seen in the first few hours. Please provide a rationale for this time point.

Authors' response: We agree with the reviewer. Originally, the protocol included an additional sample collection on day 1 following each vaccination. However, logistical challenges in coordinating the participants' return to Conakry's center for vaccination for additional sampling the day after vaccination led to the decision not to proceed with this sample collection. Many of the volunteers do not live in the city, making it difficult and potentially expensive for them to return to the vaccination center.

Fig. 2. No P values between vaccines, or vaccines and placebo are given. Please add some stats.

Authors' response: We thank the reviewer for this comment. This figure showing antibody responses in the participants of the immunological sub-study is for descriptive purposes only.

It corresponds to the main analysis of the PREVAC trial published in the NEJM in 2022 on the main trial population, where direct statistical comparisons were made between each active vaccine arm and the placebo arm at each time points post-vaccination. The aim of the ancillary study was not to repeat this analysis on a sub-sample. In addition, the PREVAC trial was methodologically designed to compare each active vaccine strategy versus the placebo arm, respectively.

Fig. 3A. Line 136 states "There were no detectable specific T-cell produced cytokines after a single dose of rVSV". On D70 there seem to be positives but this fails to reach significance, and yet the rVSV booster group, which looks very low, reaches significance. This is difficult to believe looking at the box plots. The Figure might benefit from adding the % positives mentioned in the narrative over each column.

Authors' response: We acknowledge the reviewer's perspective on the importance of this aspect in the ICS analysis. The cellular responses were subjected to quantitative analysis. Presently, there is no universally accepted threshold for clinical significance. Therefore, we have refrained from presenting the data in responder format within the statistical analysis plan, even for descriptive elucidation.

Fig. 3A. Why is the rVSV prime only group lacking from D14 plots?

Authors' response: Participants in the rVSV group received a single injection of rVSV on day 0, while participants in the rVSV-booster group received two injections: one on day 0 and another on day 56. Consequently, by day 14, participants from both groups have received only one injection of rVSV as stated in the "Methods and Materials section" (line 422, page 18). However, to better answer to the reviewer's comment, we propose to clarify the sentence as

follows: "*The participants from the rVSV and rVSV-booster groups were pooled for analyses of the time points before the boost vaccination at D56 (including Day 14)*".

Fig. 3A. The placebo group seems very high in the CD4 M12 plot. If this is an illusion because the N is greater but most are at baseline, again I recommend the % positive values are placed over each column.

Authors' response: please refer to comment above

Fig. 4. Wy were the data for different vaccines pooled? If the vaccines are analyzed separately, do any stand out as different?

Authors' response: The correlation analyses were carried out by pooling the vaccine strategies in order to increase the statistical power of the correlation analyses. Stratified analyses by vaccine arm, with very limited power, did not provide any signal for specific vaccines standing out as different. However, the correlation analysis was aimed to determine a correlation between specific responses (cellular and humoral) regardless of vaccine arms.

Fig. 5. rVSV boosting induced EBOV-specific CD4+ T-cell responses but failed to stimulate EBOV-specific CD8+ T-cell, whereas the single-dose regimen induced both. The authors suggest pre-existing immunity in the booster group blunts the recall response; can the authors speculate why this is confined to CD8 cells?

Authors' response: This is an important question that requires further investigation with a larger sample size. However, we hypothesize that this phenomenon may be confined to CD8+ T cells for several reasons: 1) Viral Vector Immunity: pre-existing immunity to the viral vector used in the rVSV booster might preferentially affect the activation and expansion of CD8+ T cells. CD8+ T cells are highly sensitive to viral infections and might be more readily suppressed by existing antibodies or memory T cells targeting the vector, preventing an effective recall response. 2) Different activation thresholds: CD8+ T cells might have different activation thresholds compared to CD4+ T cells. Pre-existing immunity could create an environment where the activation threshold for CD8+ T cells is not met, whereas CD4+ T cells, which generally have lower activation thresholds, can still be stimulated. 3) Immune regulation mechanisms: the immune system may employ regulatory mechanisms that differentially affect CD4+ and CD8+ T cells. For instance, regulatory T cells (Tregs) or other suppressive factors might be more effective at inhibiting CD8+ T cell responses in the presence of pre-existing immunity. Since these mechanisms are only hypothetical, we did not develop them in the discussion and proposed only the hypothesis of "pre-existing immunity".

Fig 5 (and other Figs). P-values: what is the rationale for NOT comparing between vaccines, only between placebo and different time points within each vaccine group.

Authors' response: We thank the reviewer for this comment. As mentioned above, the PREVAC trial was methodologically designed to compare each active vaccine strategy versus the placebo arm, respectively. The trial is not statistically powered to compare the different vaccines directly. It was also an agreement between the various consortium members not to compare active vaccines directly with each other.

Fig. 7. The legend is unreadable. Please enlarge.

Authors' response: We thank the reviewer for the suggestion. We have increased the font size of the legend

Fig. 7. Given Th2 cytokines were detected in the supernatants of peptide-restimulated PBMC, could the authors explain why they didn't perform a multifunctionality study like Fig. 3 for Th2 cells.

Authors' response: We thank the reviewer for this interesting question. Th2 cytokines play an important role in vaccine-induced responses. However, detecting them using flow cytometry is very challenging. Consequently, the majority of vaccine clinical trials focus on Th1 cytokines (IFN-γ, IL-2, and TNF), which are more readily detectable. Although it is not impossible to detect Th2 cytokines, their levels are generally lower than those of Th1 cytokines. Given that the Zaire Ebola vaccines studied in this trial induced very low levels of Th1 cytokines, we believed it would be very difficult to detect Th2 cytokines using flow cytometry. Therefore, we decided to include Th2 cytokines in the panel tested via Luminex analysis in the supernatant of stimulated cells on day 2 which is more sensitive as compared to ex vivo ICS.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have addressed my comments.

Reviewer #2:
None

Reviewer #3:
Remarks to the Author:
I am still puzzled by Fig 3. In the rebuttal a fuller figure is provided; while the Ad26-MVA d14 and d70 plots look the same as the original Fig in the paper, the rVSV booster group d70 is different, so I'm more confused. At this point, however, I think it must be me that is the problem, and since this Fig in the rebuttal is not intended for publication I'm prepared to let it go.

Overall I am satisfied with the authors' other responses and recommend publication.

Authors' responses:

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my comments.

Reviewer #3 (Remarks to the Author):

I am still puzzled by Fig 3. In the rebuttal a fuller figure is provided; while the Ad26-MVA d14 and d70 plots look the same as the original Fig in the paper, the rVSV booster group d70 is different, so I'm more confused. At this point, however, I think it must be me that is the problem, and since this Fig in the rebuttal is not intended for publication I'm prepared to let it go.

Overall I am satisfied with the authors' other responses and recommend publication.

Authors' response: The reviewer is indeed correct, and we apologize for the error attributable to a coding issue in the R program. Thanks to the reviewer's feedback, we have been able to rectify Figure 3B of the manuscript (the figure from the rebuttal letter was correct).