Decreased memory B cells frequencies in COVID-19 Delta variant vaccine breakthrough infection

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Dear Dr. Tay,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the two reviewers who agreed to evaluate your manuscript.

As you will see from their reports pasted below, while they recognize interest of your study, they also raise serious concerns, particularly regarding the overall conclusiveness of the presented data. As clear and conclusive insight into a novel, clinically relevant observation is crucial for publication in EMBO Molecular Medicine, and together with the fact that we only accept papers that receive enthusiastic support upon initial review, I am afraid that we cannot offer to consider the manuscript further.

I am sorry that I could not bring better news this time and hope that the referee comments are helpful in your continued work in this area.

Yours sincerely,

Zeljko Durdevic

Editor
EMBO Molecular Medicine

***** Reviewer’s comments *****

Referee #1 (Comments on Novelty/Model System for Author):

The authors compare patients with active COVID19 with uninfected controls. Thus, the difference in memory B cells is most likely the result of the ongoing infection, rather than the underlying cause of infection (these memory B cells are low, because they have been activated and contribute to the response, likely as plasmablast in tissue). Thus, the experimental design is not appropriate and the conclusions are not supported by the outcomes of this study.

Referee #1 (Remarks for Author):

In their manuscript, Zirui Tay et al study antibodies and memory B cells in individuals after vaccination with breakthrough infections of the SARS-CoV-2 delta variant. The topic is extremely timely and interesting as point out by the authors, because these infections do occur and it is currently unclear why.

The research question and the technical approached are very clear and appropriate. However, my main concern is the study design. The authors have included patients with a breakthrough infection within 7 days of onset of symptoms. Hence, these are patient with an active infection. These patients are compared to uninfected housemates. Same vaccination status, but not currently experiencing an infection. Thus, the authors compare immune characteristics between infected and uninfected individuals. As a result, any differences are most likely the result of the infection status rather than anything else. I do understand why this was done, but it makes all the outcomes unclear. In fact, it would make sense if there were fewer memory B cells in infected individuals as these should all have been activated and included in the response to infection.

Ideally, this study should be performed in prospectively collected samples from vaccinated individuals before any breakthrough infection and then retrospectively stratified on the basis of a subsequent infection. Then it can be addressed whether pre-existing differences underlie the risk of infection.

Because of the current fault in experimental design, the results do not support the conclusions.

Referee #2 (Comments on Novelty/Model System for Author):

Based on the data provided, I have serious concerns about the technical quality of the flow cytometry data and its analysis and interpretation. The supplementary figures show improper compensation of even easily distinguished surface markers, and the
background subtracted stimulation data suggests that at least some samples exhibited incredibly high levels of background cytokine production (>10%) that point either to issues with the samples or the gating of the flow data.

Referee #2 (Remarks for Author):

Tay et al characterise B cell, T cell and cytokine profiles of a large cohort of COVID-19 vaccine breakthrough infections and close contact controls. The cohort studied is an excellent resource and represents a large number of genuine breakthrough infections with reasonably well-matched controls. Interestingly, the authors find no differences in antibody titres between breakthrough and uninfected vaccinees, suggesting that breakthrough infection did not occur as a result of low neutralising antibody titres after vaccination.

Unfortunately, I feel the authors have missed an opportunity to incisively characterise both pre-existing and de novo T cell responses to various SARS-CoV-2 antigens in this cohort. I have serious concerns regarding the T cell flow cytometry data and its analysis. Appendix Figure S3 shows a partial gating strategy for the identification of CD4 and CD8 T cells, with apparent incorrect unmixing/compensation between CD4 and CD8. The lack of accurate unmixing and lack of representative plots showing any further T cell phenotyping or cytokine production makes it impossible to further assess the flow staining.

Furthermore, the data presented in Figure 3B are very confusing. That a median of 5% of CD8 Tcm cells would produce IL-6 in response to S peptide stimulation several months after vaccination is completely at odds with other reports of the frequency of spike-specific CD8 T cells elicited by vaccination. The presence of some data points at -10% on the graph also indicate that in several samples, the background production of IL-6 in the sample was 10% greater than the stimulated condition, which again points to either issues with the samples (such high spontaneous IL-6 production by T cells in the absence of stimulation is highly unusual) or with the gating and subsequent analysis. It is absolutely critical the authors provide FACS plots to demonstrate the cytokine staining in both stimulated and unstimulated breakthrough and control samples. For reference, what were the frequencies of IFNg+ cells in these samples? Were any positive control (mitogen) stimulations performed?

My other concern lies with the cytokine data in Figure 4. The PCA clearly shows complete overlap of the breakthrough and uninfected control cases, suggesting that the cytokine patterns analysed are not related to ongoing infection. Indeed, the vaccine breakthrough subjects in panel B do not appear to be particularly distinct from the healthy control cases, which are not even shown in the PCA analysis.
21 Oct 2021

Dear Dr Durdevic,

Thank you for your editorial work for our manuscript. We note the decision and would like to request for an appeal regarding our study. We believe that the referees are seriously mistaken in their understanding of the study, and on many occasions did not assess all the data presented. This is very concerning as it appears that the manuscript was not correctly reviewed.

We provide the following as support to explain the misinterpretation of the data. We sincerely hope that you will considering giving us the opportunity to resubmit with a complete point-by-point rebuttal and revised manuscript to further improve the clarity.

Referee #1

Referee #1’s major concern is that the lowered memory B cell signal could have been due to activation of the memory B cells upon infection, causing them to differentiate into plasmablasts. However, we wish to explain that our study also measured the plasmablast response in these patients, and our results show that this is not the case (these data were presented in the submitted manuscript).

If the memory B cells were “missing” because they had turned into plasmablasts, we should see higher levels of plasmablasts in the patients with low memory B cells, i.e. the two measure should be anti-correlated. However, we instead observe that memory B cells were positively correlated with plasmablast levels (Fig EV2b).

Thus, while we are in full agreement that we are here comparing infected individuals with uninfected individuals. We disagree that our main conclusions, including the surprising finding for memory B cells, are affected by this.

Notably, Referee #2 is in agreement with us here, noting that “the cohort studied in an excellent resource and represents a large number of genuine breakthrough infections with reasonably well-matched controls”.

Referee #2

Referee #2’s main concern was regarding the technical approach on the T cell analysis.

1. Referee #2 was concerned that there was “apparent incorrect unmixing/compensation”.

We wish to explain that a spectral flow cytometer was used here and standard automated unmixing analysis utilized. We are happy to provide representative plots showing further example flow cytometry plots, attached here in the Annex (Annex – Flow Cytometry Back-Gating).
As is clear from the plots, there is largely no drift of datapoints – and where some drift exists, it does not cause signal spillover across groups.

2. Referee #2 had concerns with Fig. 3, noting that the percentages observed by us were higher than expected.

This concern is primarily due to a difference in interpretation for Fig. 3. In Fig. 3, the Phenographs were assigned by automatic clustering, not traditional gating methods. Labels were only given afterwards to describe the cluster phenotype based on the MFI for each marker. This differs from typical gating strategies, where only a small highly distinct population is labelled positive. This was necessary to provide a qualitative description of the Phenograph clusters. This accounts for the larger percentages that we observe. To help clarify the populations in view, we have here attached flow cytometry plots for with gating for each cluster, based on the Phenograph cluster’s MFI in the highlighted parameters (Annex – Flow Cytometry Back-Gating).

**CD8 Cluster 13**

Close Contact (PTID64)- Peptide stimulated

However, it should be noted that these gatings are approximate only, since it is impossible to completely replicate the automated multiparameter gating strategy decided by the Phenograph clustering algorithm. For each cluster, we have presented representative flow cytometry plots from one vaccine breakthrough and one close contact case respectively to showcase the differences observed.

Because the same number of cells were taken for each patient, the large negative percentages in Fig. 3c may not be due to an absolute decrease in that population, but a relative decrease due to increase in other populations. Indeed, the lowest datapoint in Fig. 3c (-12.3%) corresponds to the highest datapoint in Fig. 3b (+15.3%), and the second-lowest datapoint in Fig 3c (-4.7%) corresponds to the third highest datapoint in Fig. 3b (+7.8%). To further clarify this, we have also now modified the source data to show both the percentages of cells in each Phenograph cluster for stimulated and unstimulated conditions separately, attached in the updated Source Data for Figure 3.

Positive mitogen controls were performed for all samples (stimulating with phorbol myristate acetate (PMA)), but were not presented in the manuscript since we did not
think that they were physiologically relevant. We have now added a representative set of plots showing PMA stimulation, attached in the Annex (Annex – Flow Cytometry Back-Gating).

CD8 Cluster 6

Vaccine Breakthrough (PTID52) - PMA stimulated

3. Referee #2 had concerns with the cytokine data in Fig. 4, noting that “the vaccine breakthrough subjects do not appear to be particularly distinct from the healthy control cases”.

We believe that the reviewer misunderstood the figure. Actually, this is precisely our point here: vaccination is protective to such a degree that even despite vaccine breakthrough, the inflammatory cytokine profile of these individuals remains largely similar to healthy, uninfected individuals.

This was true across the comprehensive array of cytokines that were analysed in this dataset (See Figure 4 Source Data for full list).

Notably, Referee #1 is in agreement with us here, since he was pleased with the technical approach, commenting that “the technical approaches are very clear and appropriate”.

We hope that these clarifications will set right the reviewers’ misunderstandings and provide further substantiation for our study and its high-impact conclusions.

Best regards,

Lisa F.P. Ng, PhD

Executive Director, A*STAR Infectious Diseases Labs
26th Oct 2021

Dear Dr. Tay,

Thank you for your response to the editorial decision on your manuscript entitled "Decreased memory B cells frequencies in COVID-19 Delta variant vaccine breakthrough infection". I have now carefully examined the arguments provided in your letter and discussed them with the other members of our editorial team. Additionally, I have sought external advice on the study from an expert in the field.

I am pleased to inform you that we decided to re-consider our initial decision and to invite major revision of your manuscript. Please provide detailed responses to the referee concerns and appropriately amend the manuscript to strengthen main message of the study.

Further consideration of a revision that addresses reviewers' concerns in full will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript. Please use this link to login to the manuscript system and submit your revision: https://embomolmed.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine
Referee #1 (Comments on Novelty/Model System for Author):

The authors compare patients with active COVID19 with uninfected controls. Thus, the difference in memory B cells is most likely the result of the ongoing infection, rather than the underlying cause of infection (these memory B cells are low, because they have been activated and contribute to the response, likely as plasmablast in tissue). Thus, the experimental design is not appropriate and the conclusions are not supported by the outcomes of this study.

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The research question and the technical approached are very clear and appropriate. However, my main concern is the study design. The authors have included patients with a breakthrough infection within 7 days of onset of symptoms. Hence, these are patient with an active infection. These patients are compared to uninfected housemates. Same vaccination status, but not currently experiencing an infection. Thus, the authors compare immune characteristics between infected and uninfected individuals. As a result, any differences are most likely the result of the infection status rather than anything else. I do understand why this was done, but it makes all the outcomes unclear. In fact, it would make sense if there were fewer memory B cells in infected individuals as these should all have been activated and included in the response to infection.

Ideally, this study should be performed in prospectively collected samples from vaccinated individuals before any breakthrough infection and then retrospectively stratified on the basis of a subsequent infection. Then it can be addressed whether pre-existing differences underlie the risk of infection.

Because of the current fault in experimental design, the results do not support the conclusions.

Authors’ Responses to Referee #1

• “In their manuscript, Zirui Tay et al study antibodies and memory B cells in individuals after vaccination with breakthrough infections of the SARS-CoV-2 delta variant. The topic is extremely timely and interesting as pointed out by the authors, because these infections do occur and it is currently unclear why.”

Response: We thank Referee #1 for his/her favourable evaluation of the importance of the paper topic.

• “The research question and the technical approached are very clear and appropriate.” 

Response: We thank Referee #1 for his/her favourable evaluation of our methodological approaches and their clarity.

• “… the authors compare immune characteristics between infected and uninfected individuals.”

Response: We thank Referee #1 for his/her favourable evaluation of our methodological approaches and their clarity.
“Thus, the difference in memory B cells is most likely the result of the ongoing infection, rather than the underlying cause of infection (these memory B cells are low, because they have been activated and contribute to the response, likely as plasmablast in tissue).”

Response: We thank Referee #1 for the critical examination of our paper. However, we respectfully disagree with Referee #1’s conclusion that the difference in memory B cells is the result of the ongoing infection, and that the lowered memory B cell signal could have been due to activation of the memory B cells upon infection, causing them to differentiate into plasmablasts. Notably, we measured the plasmablast response in these patients, and our results show that this was not the case. If the memory B cells were “missing” because they had turned into plasmablasts, we should observe higher levels of plasmablasts in the patients with low memory B cells, i.e. the two measures should be anti-correlated. However, we instead observe that memory B cells were positively correlated with plasmablast levels (Fig EV2b). Thus, while we are in full agreement that we are here comparing infected individuals with uninfected individuals, we disagree that our main conclusions, including the surprising finding for memory B cells, are affected by this. We have now inserted text into the manuscript (lines 168-171) to clarify this point.

- Ideally, this study should be performed in prospectively collected samples from vaccinated individuals before any breakthrough infection and then retrospectively stratified on the basis of a subsequent infection.

Response: We agree that a prospective study would provide important confirmation of the findings here. However, such a study is much more logistically challenging, especially at the scale of the number of patients analysed here. Perhaps due to this reason, previous prospective studies have focused on plasma antibody responses since plasma is much easier to process and store as compared to PBMCs. Our study is thus uniquely positioned in evaluating B and T cell responses in addition to plasma antibody responses in the context of vaccine breakthrough infection.
Referee #2 (Comments on Novelty/Model System for Author):

Based on the data provided, I have serious concerns about the technical quality of the flow cytometry data and its analysis and interpretation. The supplementary figures show improper compensation of even easily distinguished surface markers, and the background subtracted stimulation data suggests that at least some samples exhibited extremely high levels of background cytokine production (>10%) that point either to issues with the samples or the gating of the flow data.

Referee #2 (Remarks for Author):

Tay et al characterise B cell, T cell and cytokine profiles of a large cohort of COVID-19 vaccine breakthrough infections and close contact controls. The cohort studied is an excellent resource and represents a large number of genuine breakthrough infections with reasonably well-matched controls. Interestingly, the authors find no differences in antibody titres between breakthrough and uninfected vaccinees, suggesting that breakthrough infection did not occur as a result of low neutralising antibody titres after vaccination.

Unfortunately, I feel the authors have missed an opportunity to incisively characterise both pre-existing and de novo T cell responses to various SARS-CoV-2 antigens in this cohort. I have serious concerns regarding the T cell flow cytometry data and its analysis. Appendix Figure S3 shows a partial gating strategy for the identification of CD4 and CD8 T cells, with apparent incorrect unmixing/compensation between CD4 and CD8. The lack of accurate unmixing and lack of representative plots showing any further T cell phenotyping or cytokine production makes it impossible to further assess the flow staining.

Furthermore, the data presented in Figure 3B are very confusing. That a median of 5% of CD8 Tcm cells would produce IL-6 in response to S peptide stimulation several months after vaccination is completely at odds with other reports of the frequency of spike-specific CD8 T cells elicited by vaccination. The presence of some data points at -10% on the graph also indicate that in several samples, the background production of IL-6 in the sample was 10% greater than the stimulated condition, which again points to either issues with the samples (such high spontaneous IL-6 production by T cells in the absence of stimulation is highly unusual) or with the gating and subsequent analysis. It is absolutely critical the authors provide FACS plots to demonstrate the cytokine staining in both stimulated and unstimulated breakthrough and control samples. For reference, what were the frequencies of IFNg+ cells in these samples? Were any positive control (mitogen) stimulations performed?

My other concern lies with the cytokine data in Figure 4. The PCA clearly shows complete overlap of the breakthrough and uninfected control cases, suggesting that the cytokine patterns analysed are not related to ongoing infection. Indeed, the vaccine breakthrough subjects in panel B do not appear to be particularly distinct from the healthy control cases, which are not even shown in the PCA analysis.

Authors’ Responses to Referee #2

- Tay et al characterise B cell, T cell and cytokine profiles of a large cohort of COVID-19 vaccine breakthrough infections and close contact controls. The cohort studied is an excellent resource and represents a large number of genuine breakthrough infections with reasonably well-matched controls.
Response: We thank Referee #2 for the favourable evaluation of the usefulness and quality of our cohort study.

- Unfortunately, I feel the authors have missed an opportunity to incisively characterise both pre-existing and de novo T cell responses to various SARS-CoV-2 antigens in this cohort.

Response: Unfortunately, we did not examine the differences in T cell responses to the different SARS-CoV-2 antigens in this cohort, since a pooled set of SARS-CoV-2 peptides (S, S1, M and N peptides) was used rather than separate antigens. In the antibody response, very few vaccine breakthrough cases (2/55) showed (presumably de novo) responses to the N protein, and it would be interesting to see if this was similar in the T cell compartment. Unfortunately, we are unable to perform this experiment since the PBMC samples from these patients are no longer available.

- I have serious concerns regarding the T cell flow cytometry data and its analysis. Appendix Figure S3 shows a partial gating strategy for the identification of CD4 and CD8 T cells, with apparent incorrect unmixing/compensation between CD4 and CD8. The lack of accurate unmixing and lack of representative plots showing any further T cell phenotyping or cytokine production makes it impossible to further assess the flow staining.

Response: We thank the reviewer for highlighting this where in the original Appendix Fig S3, the CD8 was undercompensated relative to CD4. We had initially accepted this since the CD4+ and CD8+ T cell populations were distinct regardless. However, we took the comments serious and we have gone over our compensations and adjusted them accordingly. We have also now revised Appendix Fig S3, such that Appendix S3a shows the full gating strategy for all T cell phenotyping, and Appendix Fig S3b shows cytokine production in unstimulated, SARS-CoV-2 peptide-stimulated, and positive control PMA-stimulated conditions.
Appendix Figure S3a. Gating strategy for CD4+ and CD8+ T cells and intracellular cytokine staining. Representative flow cytometry diagrams shown for gating CD4+ and CD8+ T cells, as well as CD27/CD45RA gating for CD4+ and CD8+ T cell differentiation status.

- It is absolutely critical the authors provide FACS plots to demonstrate the cytokine staining in both stimulated and unstimulated breakthrough and control samples.

Response: We thank the reviewer for this suggestion and we have now included the flow cytometry diagrams for the cytokine staining for unstimulated, peptide-stimulated, and PMA-stimulated conditions in Appendix Fig S3b.
Appendix Figure S3b. Gating strategy for CD4+ and CD8+ T cells and intracellular cytokine staining. Representative flow cytometry diagrams shown for gating CD4+ and CD8+ T cells for intracellular cytokine staining, example flow cytometry diagrams from unstimulated (top row), SARS-CoV-2 peptide-stimulated (middle row) and PMA-stimulated (bottom row) conditions are shown.

- Furthermore, the data presented in Figure 3B are very confusing. That a median of 5% of CD8 Tcm cells would produce IL-6 in response to S peptide stimulation several months after vaccination is completely at odds with other reports of the frequency of spike-specific CD8 T cells elicited by vaccination.

Response: We note this comment and wish to explain that this confusion was caused by the original T cell analysis. The original approach assigned clusters that were labelled based on their relative fluorescence intensity, leading to much less stringent gating cut-offs than typically used for assigning positivity. This led to elevated subpopulation frequencies. Upon further consideration, we have decided to remove the dimensionality reduction and clustering approach for the T cell analysis, and have opted for a traditional gating, highlighting common cytokine/effector molecules and identifying polyfunctionality, since these are common T cell measures that have also been published in other recent papers on the T cell response against SARS-CoV-2 in vaccination and infection settings (Breton et al, 2021; Guerrera et al, 2021; Tan et al, 2021). These modifications are in lines 182-198 and lines 250-257. We sincerely believe that with the modified figures, this now removes unnecessary complexity and better streamlines the paper.

Figure 3. T cell responses in vaccine breakthrough cases and close contacts. a-d, PBMCs from vaccine breakthrough cases (n=16) and close contacts (n=26) were examined for T cell responses. PBMCs were left unstimulated (Unstim), were stimulated with pooled SARS-CoV-2 PepTivator® S, S1, M and N peptides for 6 hours (SARS-CoV-2 Peptide Stim), or non-specifically stimulated with phorbol 12-myristate 13-acetate (PMA), then assessed by high-dimensional flow cytometry. CD4+ T cells positive for intracellular staining of IFNγ (a), IL-2 (b), TNF (c), or CD8+ T cells positive for intracellular staining of granzyme B (d) are shown. e, To examine polyfunctionality of CD4+ T cells, SARS-CoV-2 peptide-stimulated IFNγ+ CD4+ T cells were further examined for co-expression of IL-2 and/or TNF, and the fraction of cells expressing IFNγ only (1 function),...
IFNγ and either IL-2 or TNF (2 functions), or IFNγ and both IL-2 and TNF (3 functions) are shown. The differentiation status of SARS-CoV-2 peptide-stimulated CD4+ T cells (left) and CD8+ T cells (right) were compared based on CD27 and CD45RA expression (Naïve: CD27+ CD45RA+; T central memory (TCM): CD27+ CD45RA-; T effector memory (TEM): CD27- CD45RA-; TEMRA: CD27- CD45RA+). The average of all vaccine breakthrough cases or close contacts are plotted respectively. P value for unpaired comparison was determined by two-tailed Mann-Whitney U test, ** P<0.01, **** P<0.0001

- Were any positive control (mitogen) stimulations performed?

Response: Yes, stimulation with phorbol 12-myristate 13-acetate (PMA) was performed in parallel as a positive control. We have now included the data for unstimulated controls and PMA-stimulated controls in Fig 3a-d and Fig EV3.

Figure EV3. Differentiation status of T cells by individual. The differentiation status of CD4+ T cells (a) and CD8+ T cells (b) in unstimulated, SARS-CoV-2 peptide-stimulated, and PMA-stimulated conditions were compared based on CD27 and CD45RA expression (Naïve: CD27+ CD45RA+; T central memory (TCM): CD27+ CD45RA-; T effector memory (TEM): CD27- CD45RA-; TEMRA: CD27- CD45RA+).

- For reference, what were the frequencies of IFNγ+ cells in these samples?

Response: Vaccine breakthrough patients showed a median of 0.26% IFNγ+ CD4+ T cells (interquartile range: 0.19%-0.81%) after peptide stimulation, and close contacts showed a median of 0.19% IFNγ+ CD4+ T cells (interquartile range: 0.08%-0.45%) after peptide stimulation. These values are in approximate agreement with the frequencies of IFNγ+ CD4+ T cells found after BNT162b2 vaccination (Guerrera et al., 2021). This data is now also shown in the revised Fig 3a.

Figure 3a. T cell IFNγ responses in vaccine breakthrough cases and close contacts. PBMCs from vaccine breakthrough cases (n=16) and close contacts (n=26) were examined for T cell responses. PBMCs were left unstimulated (Unstim), were stimulated with pooled SARS-CoV-2 PepTivator® S, S1, M and N peptides for 6 hours (SARS-CoV-2 Peptide Stim), or non-specifically stimulated with phorbol 12-myristate 13-acetate (PMA), then assessed by high-dimensional flow cytometry. CD4+ T cells positive for intracellular staining of IFNγ are shown.
- My other concern lies with the cytokine data in Figure 4. The PCA clearly shows complete overlap of the breakthrough and uninfected control cases, suggesting that the cytokine patterns analysed are not related to ongoing infection. Indeed, the vaccine breakthrough subjects in panel B do not appear to be particularly distinct from the healthy control cases, which are not even shown in the PCA analysis.

Response: We note Referee #2’s surprise at the results, and indeed, the similarity between vaccine breakthrough subjects and uninfected controls is precisely the interesting point here: vaccination appears to be protective to such a degree that even despite vaccine breakthrough, the inflammatory cytokine profile of these individuals remains largely similar to uninfected individuals. This was the result from analysis of a comprehensive panel of 45 cytokines were included for the PCA, common infection and inflammation-related cytokines such as IL-1β, TNF, IFNγ, MIP-1α, RANTES, and CXCL10 (See Figure 4 Source Data for full list). We have now also included the unvaccinated healthy controls in the PCA as well. Interestingly, the unvaccinated healthy controls cluster away from both the vaccinated group and primary infection group. The differences from the vaccinated group may be due to effects of the vaccine on innate immunity in addition to adaptive immunity, as has been reported by other groups (Arunachalam et al, 2021; Föhse et al, 2021). Nevertheless, we chose not to emphasize this in the paper as the comparison of vaccinated vs healthy unvaccinated groups is not the primary focus of the paper.

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Föhse FK, Geckin B, Overheul GJ, van de Maat J, Kılıc G, Bulut O, Dijkstra H, Lemmers H, Sarlea SA, Reijnders M et al (2021) The BNT162b2 mRNA vaccine against SARS-CoV-2 reprograms both adaptive and innate immune responses. medRxiv: 2021.2005.2003.2125620
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Tan AT, Linster M, Tan CW, Le Bert N, Chia WN, Kunasegaran K, Zhuang Y, Tham CYL, Chia A, Smith GJD et al (2021) Early induction of functional SARS-CoV-2-specific T cells associates with rapid viral clearance and mild disease in COVID-19 patients. Cell Rep 34: 108728
Dear Dr. Ng,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now heard back from a referee who agreed to re-evaluate your manuscript. In addition, I have also sought external advice on the study from an expert in the field. As you will see from the report below, the referee #2 appreciates your efforts in addressing the referees' comments but also raises considerable concerns.

As mentioned above, I have also sought further advice on the study, and our advisor reached a conclusion that "This paper ... is solid and the technologies used is up to date. I would consider its publication following last round of reviews." Therefore, I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please address all the points raised by the referee #2 in writing as limitations of the study.
2) In the main manuscript file, please do the following:
   - Correct/answer the track changes suggested by our data editors by working from the attached document.
   - Make sure that all special characters display well.
   - Add author contributions for Siti Naqiah Amrun and Bei Wang.
   - Place "Data availability" at the end of M&M section.
3) Synopsis: Please check your synopsis text and image before submission and submit their final versions with your revised manuscript. Please be aware that in the proof stage minor corrections only are allowed (e.g., typos).
4) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...
5) Press release: Please inform us as soon as possible and latest at the time of submission of the revised manuscript if you plan a press release for your article so that our publisher could coordinate publication accordingly.
6) Please be aware that we use a unique publishing workflow for COVID-19 papers: a non-typeset PDF of the accepted manuscript is published as "Just Accepted" on our website. With respect to a possible press release, we have the option to not post the "Just Accepted" version if you prefer to wait with the press release for the typeset version. Please let us know whether you agree to publication of a "Just accepted" version or you prefer to wait for the typeset version.
7) As part of the EMBO Publications transparent editorial process initiative (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts. This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication. Please note that the Authors checklist will be published at the end of the RPF.
8) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine
Reviewer’s comments

Referee #2 (Remarks for Author):

The authors have provided a revised manuscript that addresses some of the concerns of the original reviewers. In particular, the changes made to the T cell analysis in light of the issues with the original gating are appreciated, and serve to provide increased confidence in the T cell data.

With regards to the concerns raised about the B cell analysis, I am unconvinced by aspects of the revised manuscript. The authors do clearly show that the elevated plasmablast response among breakthrough cases supports the presence of active infection, and implies that reactivation of the memory B cell response has already occurred at the timepoints sampled. Under those circumstances, it is therefore confusing to conclude that differences in RBD-specific MBC reflect the immunological state prior to infection, and contribute to susceptibility to breakthrough infection. It seems equally plausible that MBC have been recruited out of the circulation due to the presence of antigen in tissues or lymph nodes.

I would note in particular that the correlation between plasmablast frequency and RBD-specific MBC frequency in EV2 is quite unconvincing - it is described in the text on line 171 as a "trend toward positive correlation", but in a sample size of n=7 with one strongly positive outlying data point, this is an overstatement. Given the number of breakthrough cases available, this analysis seems quite underpowered.
Revised Submission Referee #2

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Authors’ Responses to Referee #2 (resubmission)

- The authors have provided a revised manuscript that addresses some of the concerns of the original reviewers. In particular, the changes made to the T cell analysis in light of the issues with the original gating are appreciated, and serve to provide increased confidence in the T cell data.

Response: We thank Referee #2 for the favourable evaluation of the revised manuscript, especially for the revised T cell analysis.

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Response: We agree that the current study design does not allow us to completely rule out the possibility that MBC have been recruited out of the circulation – this would require either a prospective study design or extensive tissue sampling, both of which are out of the scope of the current study. We have previously addressed this limitation in the discussion (lines 262-266). We have now revised the manuscript to further emphasize this limitation and the possible alternative interpretation by adding the following in line 266-268: “In particular,
reduced memory B cell levels in vaccine breakthrough cases may have been due to recruitment of memory B cells out of circulation after activation by infection."

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**Response:** We were unfortunately unable to get paired plasmablast-memory B cell data for a number of the patients due to sample limitations as well as some technical difficulties. We agree that the current graph is strongly influenced by a strong positive data point; however, even if this point is removed, the remaining data still do not show a negative correlation between plasmablasts and memory B cells (see Figure EV3 with strong positive point removed; right). The lack of negative correlation is one piece of evidence against the alternate theory that MBCs are being lost via differentiation into plasmablasts. In light of this, we have now deleted the sentences regarding the trend toward positive correlation and its interpretation, and we have chosen to focus on the lack of inverse correlation instead (lines 166-169).
We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.
Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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:

- For data collected and presented in a scientific manner as reported and measured.
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D- Animal Models

- Identify the source of animals and report if they were in vitro generated and tested for pathogen contamination.
- For animal cells, provide a statement only if the cell line is the sole subject of this study and was previously validated by a recognized quality control agency.
- Provide information on animal husbandry before and after harvest.
- five (see link list at top right). According to our biosecurity guidelines, data should be deposited in one of the major public access repositories such as dbGAP.

E- Human Subjects

- Identify the committees approving the study protocol.
- Report the clinical trial registration number (at ClinicalTrials.gov), the study number and/or the source of recruitment for all clinical trials. For non-clinical trials, please provide a general description of the study population.
- Identify the committee(s) approving the study protocol.

F- Data Accessibility

- Include a "Data Accessibility" section at the end of the Materials & Methods, listing the access codes for data generated in this study and deposited in a public database (e.g., GenBank, GEO, GEO Series, GEO Console/GOAHO, Proteome data, PDB, EBML).

G- Dual use research of concern

- Provide information on any datasets that are central and integral to the study, including additional information or reference to an ancillary validation profile, e.g., antibody validation data. Please see the table at the top right of the document for all hyperlinks.