Peer Review File

Manuscript Title: Impact of circulating SARS-CoV-2 variants on mRNA vaccine-induced immunity

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

Lucas et al. analyzed the immune responses to COVID mRNA vaccination in humans with and without previous SARS-CoV-2 infection. The results showed vaccination of previously infected individuals elicited more rapid and robust immune responses, including antibodies and T cells, than those of uninfected individuals. The authors further analyzed the neutralization potencies of immune plasma against a panel of 18 authentic virus isolates, representing distinct variants of concern and variants of concern. The neutralization results indicate distinct spike mutations, particularly E484K and L452K in RBD, are responsible for reduced antibody activities. The overall study is well performed, and conclusions are supported by results.
Major points
1. The current statement on the role of N501Y in reducing neutralization activity is questionable. A detailed study on N501Y, del69/70, and E484K has been reported in https://doi.org/10.1038/s41591-021-01270-4.
2. Fig. 1d&e, the neutralization titers for group 5 should be added to indicate the trend of neutralization level over time.
3. Do the authors know the sequences of the viruses that infected the healthcare workers? This can affect the immune response to vaccination and the antibody neutralization.
4. Fig. 1b&C, the authors should explain why the level of group 5 anti-N IgG increased.
Minor points
5. Fig. 1b should be swapped with Extended Data Fig. 1b. It is important to separate the naïve vaccinated data from the previously infected vaccinated data.
6. Line 166, reference 21 should be replaced by a more appropriate reference. This is because the genotypes (i.e., D164 or G614) of the infecting viruses were not defined for the patients whose sera were tested in reference 21. An alternative reference could be NPJ Vaccines, 2021 Mar 25;6(1):44. doi: 10.1038/s41541-021-00313-8.
7. Abstract, the sentence "the latter reached…after the second vaccine dose" should be re-worded as this is not clearly described in Results.

Referee #2 (Remarks to the Author):

Lucas and colleagues compared immune responses in mRNA vaccinated and previously non-infected or SARS-CoV-2 infected individuals. In brief, they show that although vaccination triggers a faster increase in neutralizing antibodies in previously infected individuals, the peak levels are not substantially different between the two groups. Similarly, an increase in spike specific CD4 and CD8 cells was observed in both groups. Further, analysis of SARS-CoV-2 variants harboring mutations found in variants of interest/concern identified mutations associated with neutralization resistance. In addition, substantial inter-individual differences in neutralizing antibody responses were observed. Finally, evidence is provided that antibodies in vaccinated individuals previously infected with SARS-CoV-2 neutralized variants of concern with higher efficiency as compared to antibodies from vaccinated and previously non-infected individuals. In particular, the correlation between mutations in spike and neutralization sensitivity as well as the demonstration that previous infection followed by mRNA vaccination results in more robust neutralizing antibody responses are of interest. However, several points should be considered.

Major

It should be indicated in the extended data when infection of HCW was diagnosed and which variant was most likely responsible, i.e. circulating at the time of infection. Further, the implications of these data should be discussed.

It is unclear why anti-N antibody levels are increasing at 98 days post vaccination in both groups. SARS-CoV-2 infection is the only explanation for the increase in the previously non-infected group. Data must be provided to clarify this issue: Were vaccinated, previously infected individuals better protected against vaccine breakthrough infections as compared to vaccinated, not previously infected individuals?

Reference 6 reported a more pronounced impact of previous infection on induction of neutralizing antibodies upon vaccination as compared to the present study. These differences should be discussed.

Minor

The differences between previously infected and non-infected individuals shown in figures 1C and E should be subjected to statistical analysis.

Data for the two groups should be shown separately in figure 2D to allow a direct comparison.

The legend to the x-axes in figures 1C and D are missing.

Some of the viruses studied harbored mutations in M and E. Can the authors exclude that these mutations impact neutralization sensitivity?

Referee #3 (Remarks to the Author):

Lucas and colleagues analyzed the development of anti-SARS-CoV-2 antibody and T cell responses in previously infected or uninfected individuals that received mRNA vaccines to SARS-CoV-2. Importantly, they screened serum antibody responses to a panel of 16 authentic SARS-CoV-2 variants including most of the highest interest at this time. They find a hierarchy in affect on neutralization for mutations from the 16 various viral isolates that is in accord with a number of existing studies, but with several interesting novel observations. For example, the PRNT assays and modelling suggest synergistic and antagonistic combinations or independent-only affects of mutations on immune escape. The major finding of the paper supports that of previous reports that previous infection plus full prime-boost series vaccination leads to great antibody-mediated immunity.

Author Rebuttals to Initial Comments:

Referee #1

Remarks to the Author:

Lucas et al. analyzed the immune responses to COVID mRNA vaccination in humans with and without previous SARS-CoV-2 infection. The results showed vaccination of previously infected individuals elicited more rapid and robust immune responses, including antibodies and T cells, than

those of uninfected individuals. The authors further analyzed the neutralization potencies of immune plasma against a panel of 18 authentic virus isolates, representing distinct variants of concern and variants of concern. The neutralization results indicate distinct spike mutations, particularly E484K and L452K in RBD, are responsible for reduced antibody activities. The overall study is well performed, and conclusions are supported by results.

We thank the Reviewer for their supportive summary of our work.

Major points:

1. The current statement on the role of N501Y in reducing neutralization activity is questionable. A detailed study on N501Y, del69/70, and E484K has been reported in https://doi.org/10.1038/s41591-021-01270-4.

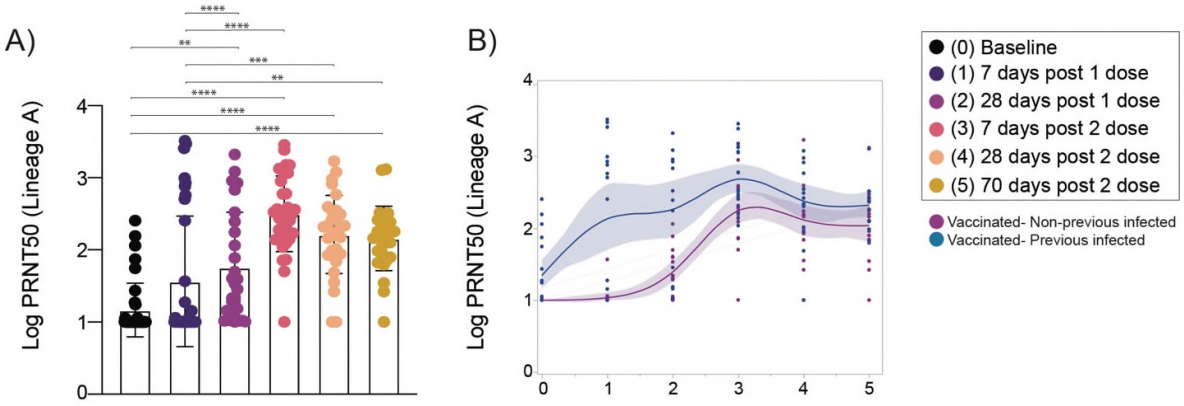
We thank the reviewer for providing a link to this study (which we now reference) and for bringing up the point about N501Y. Individually, we agree that N501Y has only a modest impact on neutralization, and we see how our text was misleading on that account. Lineages with N501Y but without other key substitutions, like L452R and E484K (B.1.1.7 + B.1.517) did not significantly impact neutralization, as previously reported. We have updated the results and discussion to minimize N501Y's individual role.

However, we find that in tandem with E484K, N501Y is potentially important. The additive effects of these two sites were not investigated in the linked manuscript, and we show a very distinct pattern of the lineages with the greatest impact on neutralization have those two substitutions. While we may still be oversimplifying the underlying causes of NAb evasion, it points to needing to better understand epistatic effects. E484 has been recognized as an important site for NAb binding, but changes to this site alone does not mean that neutralization will be severely impacted (see lineages B.1.526c and R.1). It suggests that for tracking emerging lineages with potential to escape NAbs, we should not focus entirely on single mutations, but rather specific combinations. Based on our data, new lineages with E484K and N501Y/T would be the ones to watch.

2. Fig. 1d&e, the neutralization titers for group 5 should be added to indicate the trend of neutralization level over time.

We agree with this Reviewer's suggestion regarding Figure 1D and E. We have now updated these panels (Revised Figure 1d, e) to include group 5 (70 days post 2 dose) indicating neutralization trend over time as suggested.

Reviewer's Figure 1:



a-b) Longitudinal neutralization assay using wild-type SARS-CoV-2, ancestral strain (WA1, USA). a, Neutralization titer (PRNT50) over time. Significance was assessed by One-way ANOVA corrected for multiple comparisons using Tukey's method. Boxes represent mean values ± standard deviations. b, Plasma neutralization capacity between vaccinated participants previously infected or not to SARS-CoV-2. Longitudinal data plotted over time continuously. Regression lines are shown as blue (previously infected) and purple (uninfected). Lines indicate cross-sectional averages from each group with shading representing 95% CI and are coloured accordingly. TP, vaccination time point (TP0, n = 38; TP1, n=35; TP2, n =30; TP3, n = 34; TP4, n=31; TP5, N=27). Each dot represents a single individual. ****p < .0001 ***p < .001 **p < .01*p < .05.

3. Do the authors know the sequences of the viruses that infected the healthcare workers? This can affect the immune response to vaccination and the antibody neutralization.

In the methods, we have now included the time periods during which healthcare workers tested positive by RT-qPCR and serological testing.

"Vaccinated donors were stratified in two major groups, previously infected with SARS-CoV-2 (recovered) on uninfected (naive), confirmed by RT-qPCR (10 April 2020 - 31 December 2020) and serology (2 April 2020 - 11 March 2020)."

The majority of HCWs in our study tested positive early on in 2020, with the last positive test on 31 December 2020, which is before the local emergence of the variants that we included in this study. We successfully sequenced samples from 3 out of the 15 previously infected HCWs, and all three were infected with lineage B.1.3. This confirms that previous infection was likely with generic A or B lineages that were most prevalent early on during the pandemic, which likely does not bias our findings with the panel of variants. We have now discussed this in the main text and discussion.

"Previous infection occurred between April and December 2020, and for 3 out of 15 HCWs we were able to identify the lineage of the virus, which were all B.1.3."

"Moreover, based on timing of previous infection (before the emergence of tested variants) and confirmation by sequencing (3 previous infections with B.1.3), we believe that the virus lineage of the infection likely did not have a major impact on our findings."

4. Fig. 1b&C, the authors should explain why the level of group 5 anti-N IgG increased.

We thank the reviewer for this comment and understand the concerns raised. In figure 1B and C, while we indeed observe a trend for increase in anti-N IgG, this did not reach statistical significance. We interpreted this as likely variation in our ELISA assays between experiments or due to random chance. Our ELISAs were performed in different batches using a standard curve in all plates to compare the assays. A human Anti-Spike and anti-nucleocapsid antibodies were serially diluted to generate the standard curves. However, this could still be a variation among experiments. We currently do not have additional time points or larger n to address whether this trend would eventually reach statistical significance. Individual breakthroughs cases have not been reported in either group in the time points of collection. More importantly regarding the Reviewer's point, anti-N titers in group 5 analysis are condensed and do not seem to increase due to eventual outliers, further suggesting that breakthrough indeed did not happen in our cohorts.

Minor points:

5. Fig. 1b should be swapped with Extended Data Fig. 1b. It is important to separate the naïve vaccinated data from the previously infected vaccinated data.

We thank the reviewer for this comment, but the decision of the figure representation was based on clarity and space constraint. We included Fig.1c side by side in the same scale to allow the comparison of total and vaccinated donors stratified by previous infection exposure, as proposed by the reviewer. Additionally, we used the graphs plotted over time represented with CI shadows, to allow direct comparison and visualization of statistical significance among the 2 groups.

6. Line 166, reference 21 should be replaced by a more appropriate reference. This is because the genotypes (i.e., D164 or G614) of the infecting viruses were not defined for the patients whose sera were tested in reference 21. An alternative reference could be NPJ Vaccines, 2021 Mar 25;6(1):44. doi: 10.1038/s41541-021-00313-8.

We agree with the reviewer that the suggested reference is more appropriate and have replaced it in the manuscript (see reference 21).

7. Abstract, the sentence “the latter reached…after the second vaccine dose” should be re-worded as this is not clearly described in Results.

The indicated sentence in the abstract as well as results section have been modified for clarity as suggested.

Abstract: “While previously infected individuals sustained higher antibody titers than uninfected individuals post-vaccination, the latter reached comparable levels of neutralization responses to the ancestral strain 7 days after the second vaccine dose”.

Results: We have now included group 5 in figure 1D and E.

Referee #2

Remarks to the Author:

Lucas and colleagues compared immune responses in mRNA vaccinated and previously non-infected or SARS-CoV-2 infected individuals. In brief, they show that although vaccination triggers a faster increase in neutralizing antibodies in previously infected individuals, the peak levels are not substantially different between the two groups. Similarly, an increase in spike specific CD4 and CD8 cells was observed in both groups. Further, analysis of SARS-CoV-2 variants harboring mutations found in variants of interest/concern identified mutations associated with neutralization resistance. In addition, substantial inter-individual differences in neutralizing antibody responses were observed. Finally, evidence is provided that antibodies in vaccinated individuals previously infected with SARS-CoV-2 neutralized variants of concern with higher efficiency as compared to antibodies from vaccinated and previously non-infected individuals. In particular, the correlation between mutations in

spike and neutralization sensitivity as well as the demonstration that previous infection followed by mRNA vaccination results in more robust neutralizing antibody responses are of interest. However, several points should be considered.

We thank the reviewer for her/his encouraging words and assessment.

Major:

1-It should be indicated in the extended data when infection of HCW was diagnosed and which variant was most likely responsible, i.e. circulating at the time of infection. Further, the implications of these data should be discussed.

We have now included the date range for when HCWs tested positive by RT-qPCR and serology in the methods, and have discussed the implications in the main text and discussion (see response 3 to reviewer 1). Most of the HCWs tested positive early on during the pandemic, with the last positive test on 31 December 2020, which is before the local emergence of the variants we included in the panel. We confirmed the virus lineage for 3 out of 15 infections, and identified the generic B.1.3 lineage for all three cases. This indicates that previous infection was likely with generic A or B lineages, and thus likely no impact on our findings.

Methods:

"Vaccinated donors were stratified in two major groups, previously infected with SARS-CoV2 (recovered) on uninfected (naive), confirmed by RT-qPCR (10 April 2020 - 31 December 2020) and serology (2 April 2020 - 11 March 2020)."

Main text:

"Previous infection occurred between April and December 2020, and for 3 out of 15 HCWs we were able to identify the lineage of the virus, which were all B.1.3."

Discussion:

"Moreover, based on timing of previous infection (before the emergence of tested variants) and confirmation by sequencing (3 previous infections with B.1.3), we believe that the virus lineage of the infection likely did not have a major impact on our findings."

2-It is unclear why anti-N antibody levels are increasing at 98 days post vaccination in both groups. SARS-CoV-2 infection is the only explanation for the increase in the previously non-infected group. Data must be provided to clarify this issue: Were vaccinated, previously infected individuals better protected against vaccine breakthrough infections as compared to vaccinated, not previously infected individuals?

We thank the reviewer for this comment and understand the concerns raised. In figure 1B and C, while we indeed observe a trend for increase in anti-N IgG, this did not reach statistical significance. We interpreted this as likely variation in our ELISA assays between experiments or due to random chance. Our ELISAs were performed in different batches using a standard curve in all plates to compare the assays. A human Anti-Spike and anti-nucleocapsid antibodies were serially diluted to generate the standard curves. However, this could still be a variation among experiments. We currently do not have additional time points or larger n to address whether this trend would eventually reach statistical significance. Individual breakthroughs have not been reported in either group in the time points of collection. More importantly regarding the Reviewer's point, anti-N titers in group 5 analysis are condensed and do not seem to increase due to eventual outliers, further suggesting that breakthrough indeed did not happen in our cohorts.

Regarding the review's question about breakthrough and reinfection susceptibility, we unfortunately don't have information or samples to clarify this issue.

3-Reference 6 reported a more pronounced impact of previous infection on induction of neutralizing antibodies upon vaccination as compared to the present study. These differences should be discussed.

We agree with the reviewer that although both studies indicate an increase in the neutralization antibodies upon vaccination, Stamatatos et al. reported a more pronounced induction of Nab against ancestral strain, lineage A, in previously infected individuals. However, it should be noted that our study used different strategies to measure neutralizing antibodies, fully intact authentic virus (versus pseudovirus used by Stamatatos et al.); hence these discrepancies could be related to the different assays utilized. In addition, as described in the revised discussion, our study limitations include limited and low diversity cohort that could also reflect differences among the studies. The discussion section was modified for clarity as suggested.

"The discrepancies of our results compared to other studies, including the study by Stamatatos and coworkers, may point to the importance of using fully intact authentic virus for neutralization assays to detect effects of epistasis among virus mutations on neutralization assays. Nevertheless, it remains possible that additional factors also contribute to some of the discrepancies between our observations and those of previous studies, including the presence of additional mutations in the membrane and envelope, as well as the composition of our cohorts, predominantly young Caucasian women".

Minor:

4-The differences between previously infected and non-infected individuals shown in figures 1C and E should be subjected to statistical analysis.

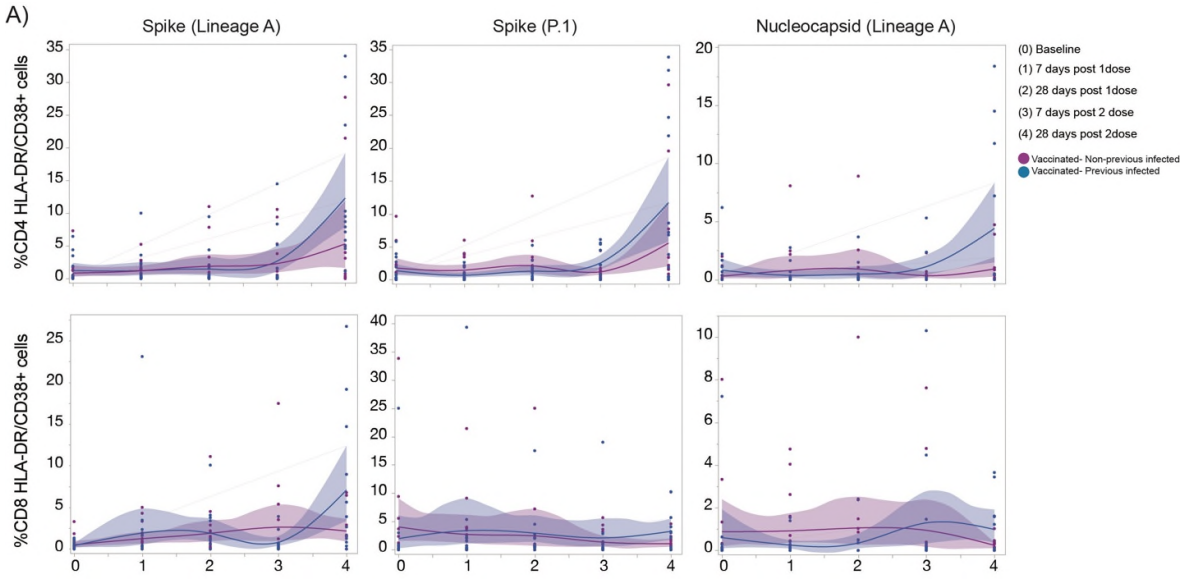
We agree with this Reviewer's point regarding the importance of showing statistical analysis.

The decision of the figure representation was based on clarity and space restraint. We included Fig.1c side by side in the same scale of Figure 1b to allow the comparison of total and vaccinated donors stratified by previous infection exposure. We have now included statical analysis as suggested. The analysis descriptions are also included in the respective figure legends.

5-Data for the two groups should be shown separately in figure 2D to allow a direct comparison.

Due to lack of significance among the groups, we opted to use the dotted colored graphs to indicate previous (blue) or not previous infected (purple) participants instead of graphs plotted continuously over time. However, to address the reviewer's point, we included a Reviewer's Figure 2, below, to direct comparison of vaccinated previous infected or non-previously infected groups.

Reviewer's Figure 2:



a) SARS-CoV-2 S-reactive CD4+ and CD8+T cells after in vitro stimulation with SARS-CoV-2 S-I and S-II peptide pools and Nucleoprotein peptides pool. S-reactive and N-reactive out of CD4+T cells (top) and CD8+T cells (bottom) over time post-vaccination. Plasma neutralization capacity between vaccinated participants previously infected or not to SARS-CoV-2. Longitudinal data plotted over time continuously. Regression lines

are shown as blue (previously infected) and purple (uninfected). Lines indicate cross-sectional averages from each group with shading representing 95% CI and are coloured accordingly.

Each dot represents a single individual. Stimulation values were subtracted from the respective non-stimulation condition. BTP, vaccination time point (TP0, n = 30; TP1, n=34; TP2, n =27; TP3, n = 27; TP4, n=24). Non-Stim, non-stimulated PBMCs. Nucleocapsid, PBMCs stimulated with SARS-CoV-2 nucleocapsid (N) protein pool derived from the ancestral lineage A virus, WA1, USA. Spike, PBMCs stimulated with SARS-CoV-2 spike (S) protein pool derived from the ancestral strain lineage A, WA1, USA. Spike (P.1), PBMCs stimulated with SARS-CoV-2 Spike (S) protein pool derived from the P.1 variant.

6-The legend to the x-axes in figures 1C and D are missing.

We apologize for the lack of clarity. These descriptions were indicated in the right top panel of the figure1, however we updated the figure 1 for better visualization of the x-axes for each panel as suggested.

7-Some of the viruses studied harbored mutations in M and E. Can the authors exclude that these mutations impact neutralization sensitivity?

Although the spike is likely the most important protein for neutralization sensitivity, we agree with the reviewer that we cannot fully exclude the potential impact of additional mutations outside the spike. We have now acknowledged this in the discussion:

“Nevertheless, it remains possible that additional factors also contribute to some of the discrepancies between our observations and those of previous studies, including the presence of additional mutations in the membrane and envelope, as well as the composition of our cohorts, predominantly young Caucasian women.”

Referee #3

Remarks to the Author:

Lucas and colleagues analyzed the development of anti-SARS-CoV-2 antibody and T cell responses in previously infected or uninfected individuals that received mRNA vaccines to SARS-CoV-2.

Importantly, they screened serum antibody responses to a panel of 16 authentic SARS-CoV-2 variants including most of the highest interest at this time. They find a hierarchy in affect on neutralization for mutations from the 16 various viral isolates that is in accord with a number of existing studies, but with several interesting novel observations. For example, the PRNT assays and modelling suggest synergistic and antagonistic combinations or independent-only affects of mutations on immune escape. The major finding of the paper supports that of previous reports that previous infection plus full prime-boost series vaccination leads to great antibody-mediated immunity.

We thank the reviewer for her/his encouraging words and assessment.

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors have appropriately addressed this reviewer's suggestions.

Referee #2 (Remarks to the Author):

The authors have adequately addressed the points raised by this reviewer. It is recommended to briefly comment (in the manuscript) on the rising levels of anti-N antibodies, which were also noted by reviewer one, and this comment could be added during a potential proofing phase. The revised manuscript is of high interest to the field.