# Peer Review File

**Manuscript Title:** SARS-CoV-2 infection induces long-lived bone marrow plasma cells in humans

## Editorial Notes:

### Redactions – unpublished data

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

## Reviewer Comments & Author Rebuttals

### Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

In their manuscript "SARS-CoV-2 induces long-lived bone marrow plasma cells in humans" Turner and collegues describe the serum titers of SARS-CoV-2 spike (S) protein specific IgG antibodies 1, 3 and 8 months after onset of symptoms in 73 COVID-19 patients experiencing a mild course of disease. They observe a more rapid decline between 1 and 3 months after onset of symptoms and a slower decline between months 3 and 8, which they interpret as a transition of antibody production from short-lived to long-lived antibody-secreting cells (ASC). In accordance, they can detect S-specific IgG or IgA ASC in bone marrow aspirates taken from 18 convalescent COVID-19 patients, 8 months after onset of clinical symptoms. Finally, while they do not detect S-specific ASC in the blood of the patients, they do detect circulating S-specific memory B lymphocytes. Here the manuscript is not informative on whether the gating strategy would have included (large) plasma cells, or whether those had been excluded by using a lymphocyte scatter gate upfront? The manuscript does add incremental evidence on the durability of the human humoral immune response to SARS-CoV-2 infection, but at this stage the evidence is much too preliminary to support the basic claim that a stable protective humoral immunity is induced in patients during the first mild COVID-19 infection.

A basic weakness of the manuscript is that it is based on a sloppy definition of "longlived" plasma cells, their location in the bone marrow, and their contribution to humoral immunity. The original reference demonstrating the persistencee of plasma cells in the bone marrow over weeks after immunization, when they are gone from the secondary lymphoid organs, is not cited (Benner, R. et al., Antibody formation in mouse bone marrow. I. Evidence for the development of plaque-forming cells in situ. Immunology 1974. 26: 247-255). Neither is the original reference cited demonstrating that those plasma cells are "long-lived", i.e. persisting as non-proliferating cells (Manz, R. A. et al., Lifetime of plasma cells in the bone marrow. Nature 1997. 388: 133-134). Remarkable that in a manuscript submitted to NATURE, an original NATURE paper is not cited. Fair enough, a follow-up paper demonstrating that plasma cells of bone marrow are not dependent on regeneration from B cells and are resistant to drugs supposed to kill proliferating cells, is cited (ref. 2). And then again, of the two concurrent papers analysing CD19- plasma cells of bone marrow, only one is cited (ref.3), the other one not (A unique population of IgG-expressing plasma cells lacking CD19 is enriched in human bone marrow. Mei et al. Blood. 2015 Mar 12;125(11):1739-48. doi: 10.1182/blood-2014-02-555169). Thus the manuscript starts upfront by confusing persistence with long-livety, not caring about demonstrating the longlivety as such. While it probably would be asked too much to demonstrate in convalescent humans resistence of ASC to anti-proliferative drugs, like cyclophosphamide or to Rituximab, the demonstration that ASC are Ki-67-negative, i.e. rest in Go of cell cycle, would have been feasible, and could have been combined with multiparametric cytometric phenotyping and identification of plasma cells as such and those expressing (intracellularly) S-protein specific IgG or IgA. This has not been done, and as they are, the data at best

demonstrate the presence of ASC in the bone marrow of convalescent COVID-19 patients, up to 7 months after onset of clinical symptoms. Whether these ASC are longlived or reflect a still ongoing immune reaction, follicular or extrafollicular, remains an open question. The data are consistent with the basic claim, as phrased in the title, but they do not prove it, nor do they exclude alternative explanations, the most extreme being that the patients still have an ongoing (acute) immune reaction.

This fuzziness continues if we compare the averaged slopes of S-specific and influenza-specific serum IgG (Fig. 1b), as deduced from measuring the titers 1, 3 and 7 months after onset. While IgG anti-influenza titers are completely stable and no "half-life" can be determined, the anti-S titers do decline between months 3 and 7, with an apparent "half-life" of about 600 days. Is this because (a) at 3 months some of the antibodies are still derived from the acute immune reaction and others already reflect a putative "long-lived" memory, or is it (b) that those ASC are "less long-lived" than the ASC secreting Influenza-specific IgG, or is it (c) just reflecting the final contraction of an acute immune reaction? The data are not informative on that, nor are these scenarios discussed in the manuscript.

The next point is the reliability of the serum antibody titrations. In Fig. 1b it appears that in quite a few individual patients serum antibody titers are dropping between month 1 and 3, and then increase again til month 7. A basic concern with these data would be (a) how reproducible are those quantifications, and (b) do the patients show different patterns of responsiveness, precluding averaging all of them and deducing "average" slopes? From the data presented, this cannot be evaluated, but it appears that either the serum IgG titrations are not robust, or indeed patients show different types of immune reactions, some of them even "secondary" immune reactions between months 3 and 7.

The most obvious drawback of the manuscript is already acknowledged by the authors themselves (lines 139ff) namely the complete lack of information on whether the antibodies analysed, esp. those produced by the ASC in the late phase of convalescence, are protective? While it would be mandatory to show that any of them are protective, it would be even more informative to learn how much of a viral challenge they can fend off. No data are provided on neutralizing capacity of the sera, not even RBD-binding, nor on secretory IgA, which would qualify to protect mucosal surfaces.

Finally, when it comes to the characterization of the circulating SARS-CoV-2-specific memory B cells and their relationship to the ASC, the data fall short of state-of-the-art options to compare their antigen-receptor repertoires, not to speak of transcriptomes. Thus the manuscript remains a bit cryptic and does not add much to our present knowledge that SARS-CoV-2 triggers in many humans a T cell-dependent immune response, including the cognate activation of B cells, and their differentiation into memory B cells and antibody-secreting cells. Protective memory is not demonstrated and the titers of serum antibodies do fade over the period of observation in most patients over the time of observation, here 7 months. And this has been demonstrated before.

Referee #2 (Remarks to the Author):

In this important paper, the persistent production of antibody to SARS-CoV-2 in patients after infection is comprehensively evaluated for up to 8-months at both the serum and, strikingly, at the cellular level in the form of long-lived bone marrow plasma cells. This work evaluates a critical and persistent question: how long will immune protection be maintained after COVID-19 exposure? An important observation for seasonal human coronaviruses is that antibody-mediated immunity wanes over time leaving people susceptible to re-infection with the same hCoV strains. While the current literature provides some insight into this issue at the serological level, the relatively short timespan since the outbreak of COVID-19 leads to limited longitudinal sampling such that one can never be certain if antibody present is from peripheral, short-lived sources such as circulating or mucosal-associated plasmablasts. Thus these observations must always be weighted with the potential that antibody may yet still wane with more time. In this paper they recruited recovered patients and demonstrate that long-lived serum responses do indeed correlate well the presence of apparent long-lived plasma cells in the bone marrow after assessing bone marrow aspirates from 18 patients. It was verified from a control, never-infected cohort that the assay

was specific and not detecting cross-reactive epitopes from endemic hCoVs, for example. While the story is convincing as is, more data ensuring that these cells are long-lived plasma cells and not recirculating plasmablasts that were in the bone marrow would be of value, such as flow cytometry verifying that the cells have terminally differentiated and so have no surface Ig, for example. Observations are also provided regarding concordant memory B cell frequencies. It would also be of value to link the bone marrow plasma cell Ig repertoire to B cell memory. To clarify, while it is completely understandable that these evaluations may be impossible to obtain based on the limited sampling and the fact that bone marrow plasma cells cannot be isolated based on antigen binding (they are cell surface Ig-negative), this data would certainly be of great interest. That said, in my opinion this is an extremely important observation to share and so should be published as soon as possible without delay if additional data is not already available.

Referee #3 (Remarks to the Author):

In this manuscript, Turner et al identify long-lived human bone marrow plasma cells that are specific for the S protein of SARS-CoV-2. These cells can be detected for at least 8 months after infection and their levels correlate with levels of anti-S antibody. The study is important and helps allay fears that SARS-CoV-2 does not induce a durable antibody response. The limitations of the study are described in good detail.

Specific comments.

There was substantial variability in the number of SARS-CoV-2 S protein-specific BMPCs identified in the 18 patients, and these cells could not be detected in 4/18 patients. Although this is a small number of patients, are there any clinical or laboratory characteristics of the patients that could explain the variability of the response?
-Which of the patients described in Extended Data Table 1 underwent BM biopsy?

**Author Rebuttals to Initial Comments:**

## Response to Reviewers:

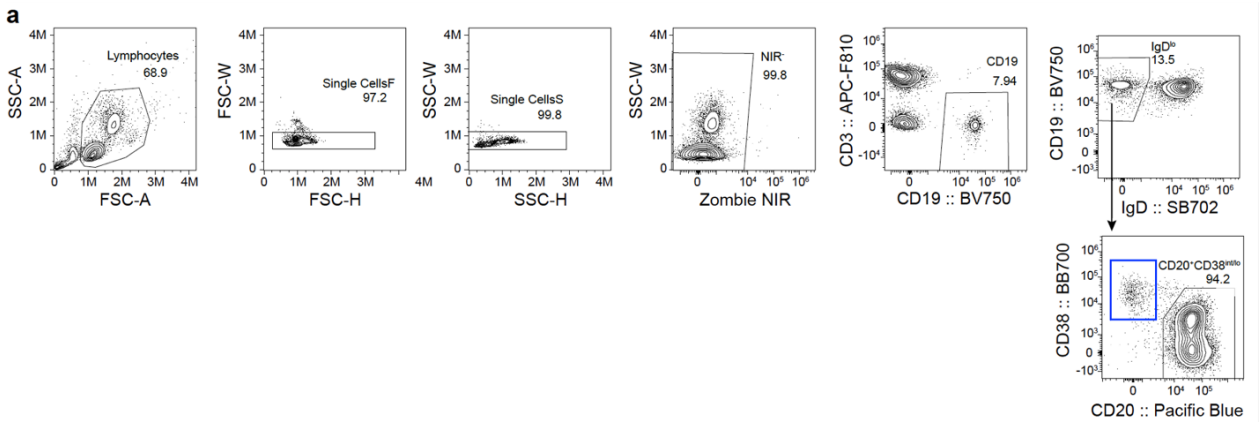
Reviewer #1:

1) In their manuscript "SARS-CoV-2 induces long-lived bone marrow plasma cells in humans" Turner and collogues describe the serum titers of SARS-CoV-2 spike (S) protein specific IgG antibodies 1, 3 and 8 months after onset of symptoms in 73 COVID-19 patients experiencing a mild course of disease. They observe a more rapid decline between 1 and 3 months after onset of symptoms and a slower decline between months 3 and 8, which they interpret as a transition of antibody production from short-lived to long-lived antibody-secreting cells (ASC). In accordance, they can detect S-specific IgG or IgA ASC in bone marrow aspirates taken from 18 convalescent COVID-19 patients, 8 months after onset of clinical symptoms. Finally, while they do not detect S-specific ASC in the blood of the patients, they do detect circulating S-specific memory B lymphocytes.

*We appreciate the concise summary of our data and overall, we greatly appreciate Reviewer #1's constructive comments and critiques that will help clarify the importance of the study.*

2) Here the manuscript is not informative on whether the gating strategy would have included (large) plasma cells, or whether those had been excluded by using a lymphocyte scatter gate upfront?

*This is an important point. We already had addressed this concern as we showed in the **Extended Data Fig. 1a** of the submitted manuscript (also, included below) that the PBMC lymphocyte gating did include large plasma cells. This is evidenced by the plasmablasts (CD19+, IgD-, CD38hi, CD20lo) we detect in the blue gate below. We will highlight this more clearly in the main text.*




3) The manuscript does add incremental evidence on the durability of the human humoral immune response to SARS-CoV-2 infection, but at this stage the evidence is much too preliminary to support the basic claim that a stable protective humoral immunity is induced in patients during the first mild COVID-19 infection.

*Respectfully, we disagree with the Reviewer. This is the first and, so far, only report that establishes the generation of an antigen-specific bone marrow plasma cell pool 7-8 months aftermild SARS-CoV-2 infection in humans. These cells are totally absent from the circulation and normal sampling by phlebotomy at that time point. This is a technically challenging experiment (obtaining bone marrow biopsies from **18** volunteers who had been infected with SARS-CoV-2) and we feel, not an incremental evidence by any measure.*

4) A basic weakness of the manuscript is that it is based on a sloppy definition of "long-lived" plasma cells, their location in the bone marrow, and their contribution to humoral immunity. The original reference demonstrating the persistence of plasma cells in the bone marrow over weeks after immunization, when they are gone from the secondary lymphoid organs, is not cited (Benner, R. et al., Antibody formation in mouse bone marrow. I. Evidence for the development of plaque-forming cells in situ. Immunology 1974. 26: 247-255). Neither is the original reference cited demonstrating that those plasma cells are "long-lived", i.e. persisting as non-proliferating cells (Manz, R. A. et al., Lifetime of plasma cells in the bone marrow. Nature 1997. 388: 133-134). Remarkable that in a manuscript submitted to NATURE, an original NATURE paper is not cited. Fair enough, a follow-up paper demonstrating that plasma cells of bone marrow are not dependent on regeneration from B cells and are resistant to drugs supposed to kill proliferating cells, is cited (ref. 2). And then again, of the two concurrent papers analyzing CD19- plasma cellsof bone marrow, only one is cited (ref.3), the other one not (A unique population of IgG-expressingplasma cells lacking CD19 is enriched in human bone marrow. Mei et al. Blood. 2015 Mar 12;125(11):1739-48. doi: 10.1182/blood-2014-02-555169).

*We agree with this comment and thank the reviewer for pointing out the lack of a clear and comprehensive background for the work we are presenting. We will clarify the definition of long-lived plasma cells in the Introduction section, place our work in the proper context of the broader field, and add the appropriate citations.*

5) Thus, the manuscript starts upfront by confusing persistence with long-livety, not caring about demonstrating the longlivety as such. While it probably would be asked too much to demonstrate in convalescent humans resistance of ASC to anti-proliferative drugs, like cyclophosphamide or to Rituximab, the demonstration that ASC are Ki-67-negative, i.e. rest in Go of cell cycle, would have been feasible, and could have been combined with multiparametric cytometric phenotyping and identification of plasma cells as such and those expressing (intracellularly) S-protein specific IgG or IgA.

*This is an important point. We do have preserved bone marrow specimens from the participants that we can use to examine the expression of suggested proliferation markers in plasma cells from peripheral blood and bone marrow. The suggestion to identify S-specific plasma cells by intracellular staining is an excellent one. However, it is important to realize that the frequency of S-specific IgG+ plasma cells is relatively low, with some individuals having these cells at 1 in 5,000 or 1 in 10,000 total plasma cells as shown in **Fig. 2b**. The frequency of S-specific IgA+ plasma cells is even lower. To address this concern, we will perform an extensive multiparametriccytometric phenotyping on plasma cells from bone marrow and peripheral blood, including markers of proliferation. We will also attempt to identify S-specific cells among bone marrow cellplasma cells.*

6) This has not been done, and as they are, the data at best demonstrat[illegible] the presence of ASC in the bone marrow of convalescent COVID-19 patients, up to 7 months [illegible] after onset of clinical symptoms. Whether these ASC are longlived or reflect a still ongoing [illegible] immune reaction, follicular or extrafollicular, remains an open question. The data are consistent with the basic claim, as phrased in the title, but they do not prove it, nor do they exclude alterna[illegible]tive explanations, the most extreme being that the patients still have an ongoing (acute) immune [illegible]

*We again respectfully disagree with the Reviewer. We believe that the identified population is partof bona fide long-lived plasma cell pool as evidenced by:*

1) *All of our convalescent participants have experienced a mild SARS-CoV-2 infection, with only 1 out of 18 participants from whom we sampled bone marrow needing hospitalization. In addition, we examined these bone marrow specimens 7-8 months after symptom onset. Therefore, we believe that this precludes the possibility of any of the patients having an "acute" response.*

***Ellebedy, Ali***
*2021-01-20 17:20:00*
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Estimated time needed: 2-3 weeks.

2) *In the participants who donated bone marrow specimens at the later time point, we did examine their PBMCs for possible evidence of an actively ongoing B cell response. We did not detect any S-specific plasmablasts (by ELISpot or flow cytometry) or any S-specific recently activated B cells (CD38int CD71hi – flow cytometry).*

7) This fuzziness continues if we compare the averaged slopes of S-specific and influenza-specific serum IgG (Fig. 1b), as deduced from measuring the titers 1, 3 and 7 months after onset. While IgG anti-influenza titers are completely stable and no "half-life" can be determined, the anti-S titers do decline between months 3 and 7, with an apparent "half-life" of about 600 days. Is this because (a) at 3 months some of the antibodies are still derived from the acute immune reaction and others already reflect a putative "long-lived" memory, or is it (b) that those ASC are "less long-lived" than the ASC secreting Influenza-specific IgG, or is it (c) just reflecting the final contraction of an acute immune reaction? The data are not informative on that, nor are these scenarios discussed in the manuscript.

*We believe that the one of the scenarios described by the Reviewer (at 3 months some of the antibodies are still derived from the acute immune reaction yet others already reflect a "long-lived" plasma cell response) is most likely what is causing the differences we observed in decay slopes. We do not have any data to support the existence of intrinsic differences between S-specific and influenza-specific plasma cells. We will clarify this point in the Discuss*

**Ellebedy, Ali**
*2021-01-20 04:31:00*
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Response: We will further clarify this point in the discussion section. Estimated time: 1 week

8) The next point is the reliability of the serum antibody titrations. In Fig. 1 [illegible] that in quitea few individual patients serum antibody titers are dropping between m[illegible]onth 1 and 3, and then increase again till month 7. A basic concern with these data would be [illegible](a) how reproducible are those quantifications, and (b) do the patients show different patterns [illegible]of responsiveness, precluding averaging all of them and deducing "average" slopes? Fro[illegible] cannot be evaluated, but it appears that either the serum IgG titration[illegible] patients show different types of immune reactions, some of them even "secondary" immune reactions between months 3 and 7.

*We thank the reviewer for bringing up this important point. We have checked the individual slopes.In 54/73 (74%) of the participants, the slopes followed the pattern shown by the average (negativeslope from month 1 to month 3-4 and attenuated negative slope from month 3-4 to month 7-8). Inthree participants (4%), the slopes exhibited the intriguing pattern noted by the reviewer (negativeslope in first period followed by a positive slope in the second). This could be stochastic noise, orthey could be real patterns that imply possible re-infection (which is certainly plausible given the number of infections in the United States). We could certainly address these points experimentallyby repeating these measures and looking at different isotypes, such as IgM. Thus, to [illegible]some of the measurements among the distinct subgroups to ensure th[illegible] We will also perform a rigorous statistical analysis and analyze in[illegible] longitudinal changes in anti-SARS-CoV-2-D IgM levels.*

**Ellebedy, Ali**
*2021-01-20 13:39:00*
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Response: Repeat measurements to examine the reproducibility of the data. Perform a rigorous statistical analysis of the data to examine the

9) The most obvious drawback of the manuscript is already acknowledg[illegible]ed by the authors themselves (lines 139ff) namely the complete lack of information on w[illegible] analysed, esp. those produced by the ASC in the late phase of conva[illegible] While it would be mandatory to show that any of them are protective, [illegible]it would be even more informative to learn how much of a viral challenge they can fend off. No data are provided on neutralizing capacity of the sera, not even RBD-binding, nor on secretory IgA, which would qualify to protect mucosal surfaces.

*We agree with the reviewer that duration of protective antibody responses is a critical point. We will experimentally address their concerns and evaluate serum neutralization, RBD-binding and IgA responses. However, to address the protective potential of any of the S-specific plasma cells,*

*we would need to express the corresponding monoclonal antibody from that cell and assess its protective potential in vivo. Apart from being outside the scope of this manuscript, technically thisis very challenging task because of the extremely low frequency of these cells, and the fact that we cannot single cell sort these cells using labeled S probes due to lack of surface Ig, which is in agreement with Reviewer 2 comments.*

10) Finally, when it comes to the characterization of the circulating SA
B cells and their relationship to the ASC, the data fall short of state-of
their antigen-receptor repertoires, not to speak of transcriptomes. Thu
bit cryptic and does not add much to our present knowledge that SAR
humans a T cell-dependent immune response, including the cognate
their differentiation into memory B cells and antibody-secreting cells.
demonstrated, and the titers of serum antibodies do fade over the per
patients over the time of observation, here 7 months. And this has been demonstrated before.

**Ellebedy, Ali**
*2021-01-20 05:43:00*
~~the manuscript remains a~~
Response: We will assess the neutralizing activity of the sera, RBD-binding, and serum IgA responses.
Estimated time: 2-3 weeks

*Comparing S-specific antigen-receptor B cell repertoires between circulating memory B cells and bone marrow plasma cells is an excellent point that also was raised by Reviewer 2 below. However, and as acknowledged by Reviewer 2, this is technically challenging because bone marrow plasma cells cannot be isolated based on antigen binding. Our main goal from the memory B cell figure is to show that we can also detect a circulating S-specific resting memory Bcell compartment. We agree with the Reviewer that this point has been reported by multiple groups already, and it is not the main focus of our study.*

**Reviewer #2:**

In this important paper, the persistent production of antibody to SARS-CoV-2 in patients after infection is comprehensively evaluated for up to 8-months at both the serum and, strikingly, at the cellular level in the form of long-lived bone marrow plasma cells. This work evaluates a critical and persistent question: how long will immune protection be maintained after COVID-19 exposure? An important observation for seasonal human coronaviruses is that antibody-mediatedimmunity wanes over time leaving people susceptible to re-infection with the same hCoV strains.While the current literature provides some insight into this issue at the serological level, the relatively short time span since the outbreak of COVID-19 leads to limited longitudinal sampling such that one can never be certain if antibody present is from peripheral, short-lived sources suchas circulating or mucosal-associated plasmablasts. Thus, these observations must always be weighted with the potential that antibody may yet still wane with more time. In this paper they recruited recovered patients and demonstrate that long-lived serum responses do indeed correlate well the presence of apparent long-lived plasma cells in the bone marrow after assessingbone marrow aspirates from 18 patients. It was verified from a control, never-infected cohort thatthe assay was specific and not detecting cross-reactive epitopes from endemic hCoVs, for example.

*We appreciate the supportive summary of the work.*

While the story is convincing as is, more data ensuring that these cells are long-lived plasma cells and not recirculating plasmablasts that were in the bone marrow would be of value, such as flow cytometry verifying that the cells have terminally differentiated and so have no surface Ig, for example.

*We thank the Reviewer for highlighting this important point that is consistent with Reviewer 1's point #5 (see above). As described above, we will thoroughly address this point by performing an extensive multiparametric cytometric phenotyping on plasma cells from bone marrow and peripheral blood, including markers of maturation and proliferation.*

Observations are also provided regarding concordant memory B cell frequencies. It would also be of value to link the bone marrow plasma cell Ig repertoire to B cell memory. To clarify, while itis completely understandable that these evaluations may be impossible to obtain based on the limited sampling and the fact that bone marrow plasma cells cannot be isolated based on antigenbinding (they are cell surface Ig-negative), this data would certainly be of great interest.

*We agree with the Reviewer regarding how valuable yet technically challenging such an analysis would be (also discussed in our response to Reviewer 1 #9).*

That said, in my opinion this is an extremely important observation to share and so should be published as soon as possible without delay if additional data is not already available.

*We greatly appreciate the supportive comments about the importance and impact of the study.*

**Reviewer #3:**

In this manuscript, Turner et al identify long-lived human bone marrow plasma cells that are specific for the S protein of SARS-CoV-2. These cells can be detected for at least 8 months afterinfection and their levels correlate with levels of anti-S antibody. The study is important and helpsallay fears that SARS-CoV-2 does not induce a durable antibody response. The limitations of thestudy are described in good detail.

*We appreciate the supportive summary of the work.*

Specific comments.

There was substantial variability in the number of SARS-CoV-2 S protein-specific BMPCs identified in the 18 patients, and these cells could not be detected in 4/18 patients. Although thisis a small number of patients, are there any clinical or laboratory characteristics of the patients that could explain the variability of the response?

*We agree with the Reviewer that this is an intriguing point. We have examined multiple parameters, including age, sex, co-morbidities, concurrent medications, severity and duration of symptoms, and the time span between onset of symptoms and collection of the bone marrow aspirate and could not find a trend that would explain the lack of detectable bone marrow plasmacells in those four participants compared to the rest. As noted by the Reviewer, this could be justan assay limit of detection issue, as we proposed in the discussion.*

Which of the patients described in Extended Data Table 1 underwent BM biopsy?

*This is a great suggestion. A separate Extended Table for those participants will be added.*

**Ellebedy, Ali**

*2021-01-20 11:57:00*

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Response: We will add a separate table detailing the characteristics of the 18 participants from whom we analyzed bone marrow specimens. Estimated

**Reviewer Reports on the First Revision:**

N/A

**Author Rebuttals to First Revision:**

We would like to thank the editor and reviewers for their time and careful consideration of our manuscript. The comments provided were very helpful and we feel that completing the recommended experiments has improved the manuscript. Additionally, the experiments proposed have allowed us to highlight new details about the bone marrow long-lived plasma cell (LLPC) response to SARS-CoV-2 infection. Broadly, the new data provide evidence for:

1. Extended durability of serum anti-spike (S) IgG titers that are induced after mild SARS-CoV-2

infection in humans. This was shown by analyzing serum specimens collected from SARS-CoV-2 convalescent patients up to 11 months after symptoms onset (*n*=42).

2. The stability of the observed bone marrow LLPC compartment induced after SARS-CoV-2 infection. This was demonstrated by showing a negligible change in frequency of S-specific IgG-secreting bone marrow plasma cells in paired bone marrow aspirates and serum specimens collected at 7 and 11 months after symptom onset (*n*=5).
3. S-specific bone marrow LLPCs observed in SARS-CoV-2 convalescent patients are quiescentas evidenced by their lack of expression of proliferation markers, such as Ki67. This is in contrastto recently generated S-specific plasmablasts.
4. Antibodies that are capable of neutralizing authentic SARS-CoV-2 and recently emerging variants can be detected in serum from convalescent patients isolated up to 7 months after symptom onset, indicating that at least some of the LLPCs are encoding neutralizing antibodies.

Below we have provided our responses (in *blue*) to reviewers' comments detailing the ways in which our revised manuscript addresses their concerns.

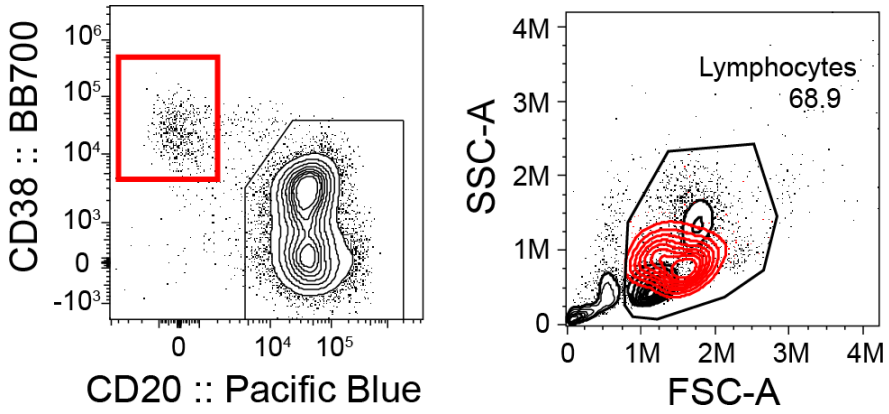
**Reviewer #1:**

1) In their manuscript "SARS-CoV-2 induces long-lived bone marrow plasma cells in humans" Turner and collogues describe the serum titers of SARS-CoV-2 spike (S) protein specific IgG antibodies 1, 3 and 8 months after onset of symptoms in 73 COVID-19 patients experiencing a mild course of disease. They observe a more rapid decline between 1 and 3 months after onset of symptoms and a slower decline between months 3 and 8, which they interpret as a transition of antibody production from short-lived to long-lived antibody-secreting cells (ASC). In accordance, they can detect S-specific IgG or IgA ASC in bone marrow aspirates taken from 18 convalescent COVID-19 patients, 8 months after onset of clinical symptoms. Finally, while they do not detect S-specific ASC in the blood of the patients, they do detect circulating S-specific memory B lymphocytes.

*We appreciate the concise summary of our data. Overall, we greatly appreciate Reviewer #1's constructive comments and critiques that helped clarify the importance of our study.*

2) Here the manuscript is not informative on whether the gating strategy would have included (large) plasma cells, or whether those had been excluded by using a lymphocyte scatter gate upfront?

*We agree with the Reviewer that this is an important point. As shown in the figure below, the PBMC lymphocyte gating we used did include large plasma cells. This is evidenced by the plasmablasts (CD19+, IgD-, CD38hi, CD20lo) we detect in the red gate below (from a 7m timepoint from our memory B cell analysis) as well as detection of the plasmablasts induced one week after vaccination we use for comparison to BMPCs in Fig. 3. We have now highlighted thismore clearly in the main text (line 126).*




**Reviewer Figure 1. Scatter properties of circulating plasmablasts.**

Representative plot showing CD20$^{lo}$ CD38$^{+}$ plasmablasts, pregated on IgD$^{lo}$ CD19$^{+/lo}$ CD3$^{-}$ live singlet lymphocytes (*left panel, red gate*) and their forward and side scatter (*right panel, red contours*) among ungated events.

3) The manuscript does add incremental evidence on the durability of the human humoral immune response to SARS-CoV-2 infection, but at this stage the evidence is much too preliminary to support the basic claim that a stable protective humoral immunity is induced in patients during the first mild COVID-19 infection.

*We agree with the Reviewer that at the time of the first submission of this manuscript there was minimal evidence for protection against re-infection. Over the last few months, multiple epidemiological studies have documented the enhanced protection against re-infection among SARS-CoV-2 convalescent patients. For example, a recently published study (The SIREN cohort) demonstrated that ".. previous history of SARS-CoV-2 infection was associated with an 84% lower risk of infection, with median protective effect observed 7 months following primary infection." (Lancet, DOI: https://doi.org/10.1016/S0140-6736(21)00675-9). Our study is so far the only report that establishes the generation of an antigen-specific bone marrow plasma cell pool 7-8 months after mild SARS-CoV-2 infection in humans. These cells are totally absent from the circulation at that time point. We also show that these cells are distinct from recently generated plasmablasts. Given the challenging nature of these specimens and analyses, we think our studies provide more than just an incremental evidence for the durable humoral immune response elicited by SARS-CoV-2 infection in humans.*

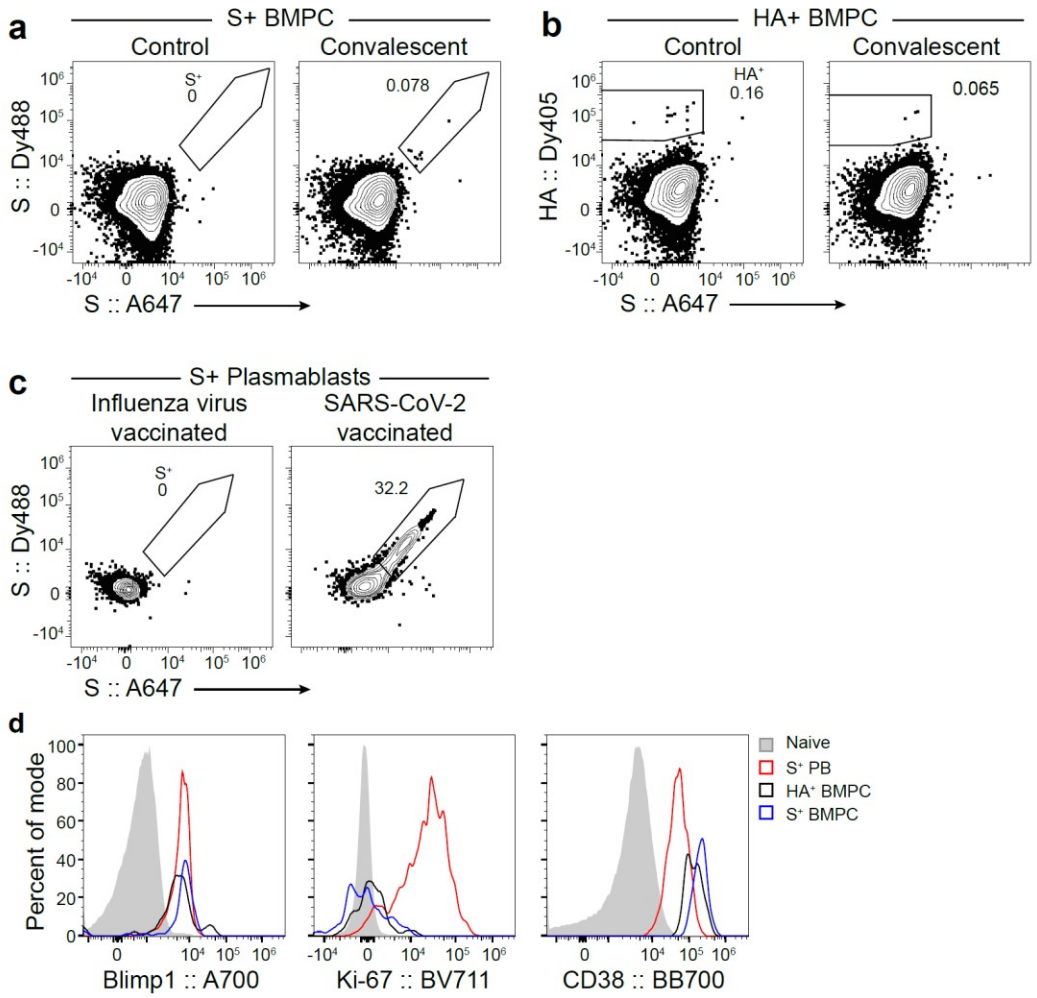
4) A basic weakness of the manuscript is that it is based on a sloppy definition of "long-lived" plasma cells, their location in the bone marrow, and their contribution to humoral immunity. The original reference demonstrating the persistence of plasma cells in the bone marrow over weeks after immunization, when they are gone from the secondary lymphoid organs, is not cited (Benner, R. et al., Antibody formation in mouse bone marrow. I. Evidence for the development of plaque-forming cells in situ. Immunology 1974. 26: 247-255). Neither is the original reference cited demonstrating that those plasma cells are "long-lived", i.e. persisting as non-proliferating cells (Manz, R. A. et al., Lifetime of plasma cells in the bone marrow. Nature 1997. 388: 133-134). Remarkable that in a manuscript submitted to NATURE, an original NATURE paper is not cited. Fair enough, a follow-up paper demonstrating that plasma cells of bone marrow are not dependent on regeneration from B cells and are resistant to drugs supposed to kill proliferating cells, is cited (ref. 2). And then again, of the two concurrent papers analyzing CD19- plasma cells

of bone marrow, only one is cited (ref.3), the other one not (A unique population of IgG-expressing plasma cells lacking CD19 is enriched in human bone marrow. Mei et al. Blood. 2015 Mar 12;125(11):1739-48. doi: 10.1182/blood-2014-02-555169).

*We agree with this comment and thank the Reviewer for pointing out the lack of a clear and comprehensive background for the work we are presenting. Also, we apologize for failing to place our work in the proper context of the field's history, including missing some key citations. We have clarified the definition of long-lived plasma cells as non-replicating, antigen-specific plasma cells that are detected in bone marrow long after the disappearance of the antigen and in the absenceof circulating plasmablasts directed against said antigen (line 59). In addition, we placed our workin the proper context of the broader field and added the appropriate citations.*

5) Thus, the manuscript starts upfront by confusing persistence with long-livety, not caring about demonstrating the longlivety as such. While it probably would be asked too much to demonstrate in convalescent humans resistance of ASC to anti-proliferative drugs, like cyclophosphamide or to Rituximab, the demonstration that ASC are Ki-67-negative, i.e. rest in Go of cell cycle, would have been feasible, and could have been combined with multiparametric cytometric phenotyping and identification of plasma cells as such and those expressing (intracellularly) S-protein specific IgG or IgA.

*This is an excellent suggestion. We used preserved bone marrow specimens from the SARS- CoV-2 convalescent patients to examine the expression of Ki-67 in spike-specific plasma cells in comparison to bone marrow plasma cells that are specific to influenza virus hemagglutinin (HA) and recently generated spike-specific circulating plasmablasts. As suggested by the Reviewer, we identified antigen-specific plasma cells by intracellular staining. The frequency of S+ bone marrow plasma cells was low (consistent with the ELISpot data). Importantly S+ (and HA+) bonemarrow plasma cells are quiescent as indicated by lack of Ki-67 expression in contrast to recentlygenerated S+ blood plasmablasts. In addition, the bone marrow plasma cell populations expressed higher levels of CD38 compared to circulating plasmablasts. We have added these findings to the manuscript as Fig. 3, and they are below as Reviewer Fig. 2.*




**Reviewer Figure 2. Identification of antigen-specific BMPCs by flow cytometry**

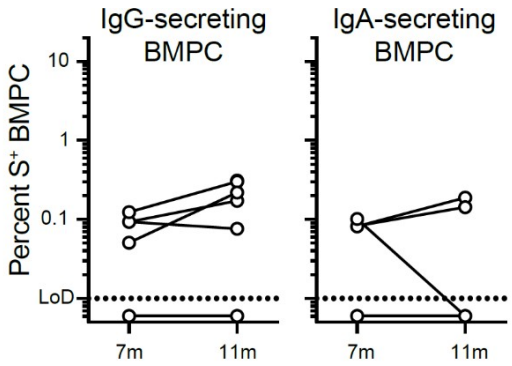
**a, b,** Representative plots of intracellular S (a) and influenza virus hemagglutinin (b) staining of BMPCs (pregated CD20$^{lo}$ CD38$^{+}$ IgD$^{lo}$ CD19$^{+/lo}$ CD3$^{-}$ live singlets) from control (*left*) and convalescent (*right*) bone marrow. **c**, Representative plots of S intracellular staining in IgD$^{lo}$ CD20$^{lo}$ CD38$^{+}$ CD19$^{+}$ CD3$^{-}$ live singlet plasmablasts in PBMC 1 week after seasonal influenza virus or SARS-CoV-2 vaccination. **d,** Histographs of Blimp1 (*left*), Ki-67 (*center*), and CD38 (*right*) staining on S$^{+}$ (blue) and HA$^{+}$ (black) BMPC 7m post symptom onset and S$^{+}$ plasmablasts (red), and naïve B cells (grey) from healthy donor PBMC 1 week after SARS-CoV-2 S immunization.

6) This has not been done, and as they are, the data at best demonstrate the presence of ASC in the bone marrow of convalescent COVID-19 patients, up to 7 months after onset of clinical symptoms. Whether these ASC are longlived or reflect a still ongoing immune reaction, follicular or extrafollicular, remains an open question. The data are consistent with the basic claim, as phrased in the title, but they do not prove it, nor do they exclude alternative explanations, the most extreme being that the patients still have an ongoing (acute) immune reaction.

*Two pieces of data address the important concerns raised by the Reviewer here.*

1) *The frequency of S+ bone marrow plasma cells experienced minimal change in participants sampled a second time at 11 months after symptom onset (3-4 months after first bone marrow aspiration), excluding the possibility that the initial levels were secondary to an ongoing extrafollicular response. These data are shown in Fig. 2c and below as Reviewer Fig. 3)*
2) *All of our convalescent participants experienced mild SARS-CoV-2 infection, with only 1 out of 18 participants from whom we sampled bone marrow needing hospitalization. We examined these bone marrow specimens 7-8 months after symptom onset. And at that time point, we did examine their PBMCs for possible evidence of an actively ongoing plasmablast response. We did not detect any S+ plasmablasts (by ELISpot or flow cytometry) or any S+ recently activated B cells (CD38int CD71hi by flow cytometry).*

*These data argue against the possibility of any of the patients having an "acute" response and we, therefore, believe that the identified S+ plasma cell population is part of bona fide long-lived plasma cell pool.*




**Reviewer Figure 3. S-binding BMPC frequencies in paired samples 7- and 11-months post symptom onset**

Paired frequencies of BMPC secreting IgG (*left*) or IgA (*right*) specific for S from convalescent participants 7m and 11m post symptom onset. Each symbol represents one sample, with lines connecting paired samples (n=6). Dotted line indicates limit of detection.

7) This fuzziness continues if we compare the averaged slopes of S-specific and influenza-specific serum IgG (Fig. 1b), as deduced from measuring the titers 1, 3 and 7 months after onset. While IgG anti-influenza titers are completely stable and no "half-life" can be determined, the anti-S titers do decline between months 3 and 7, with an apparent "half-life" of about 600 days. Is this because (a) at 3 months some of the antibodies are still derived from the acute immune reaction and others already reflect a putative "long-lived" memory, or is it (b) that those ASC are "less long-lived" than the ASC secreting Influenza-specific IgG, or is it (c) just reflecting the final contraction of an acute immune reaction? The data are not informative on that, nor are these scenarios discussed in the manuscript.

*We believe that the one of the scenarios described by the Reviewer (at 3 months some of the antibodies are still derived from the acute immune reaction while others are from the "long-lived" plasma cell response) is most likely what caused the differences we observed in decay slopes. We do not have any data to support the existence of intrinsic differences between S-specific and influenza-specific plasma cells. We have emphasized this point and added the alternative scenarios proposed by the Reviewer in the Discussion (line 176).*

*More broadly, we reevaluated our strategy for analysis of serum antibody durability to place more emphasis on our observation of a larger decrease in antibody titers between 1 and 4 months compared to the decreases between 3- and 11-months post symptom onset, rather than assigning discrete half-lives to the artificially imposed intervals between sample collection timepoints. We feel this more clearly communicates the larger point that in the majority of patients, although serum antibody titers wane relatively quickly from an early peak, they remain detectable 11m aftermild SARS-CoV-2 infection and are likely maintained by long-lived BMPCs.*

8) The next point is the reliability of the serum antibody titrations. In Fig. 1b it appears that in quite a few individual patients serum antibody titers are dropping between month 1 and 3, and then increase again till month 7. A basic concern with these data would be (a) how reproducible are those quantifications, and (b) do the patients show different patterns of responsiveness, precluding averaging all of them and deducing "average" slopes? From the data presented, this cannot be evaluated, but it appears that either the serum IgG titrations are not robust, or indeed patients show different types of immune reactions, some of them even "secondary" immune reactions between months 3 and 7.

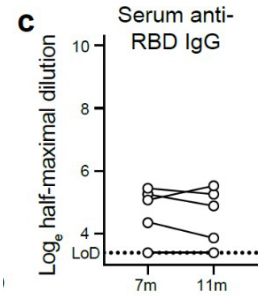
*We thank the Reviewer for bringing up this important point. We added 4 additional participants to the study and re-measured spike-binding IgG titers in selected participants; all were consistent with previously measured titers. Additionally, we re-analyzed the individual slopes. In 58/77 (75%)of the participants, the slopes followed the pattern shown by the average (negative slope from month 1 to month 3-4 and attenuated negative slope from month 3-4 through month 7 or 11). In three participants (4%), the slopes exhibited the intriguing pattern noted by the Reviewer(negative slope in first period followed by a positive slope in the second). This could be stochasticnoise or could represent real patterns that imply possible higher net binding affinity as early plasmablast-derived antibodies are replaced by those from affinity-matured LLPCs or increases in antibody concentration from reencounter with the virus (which is plausible given the number ofinfections in the United States, although none of the participants have tested positive again). Wehave added these points to the Discussion (line 169).*

9) The most obvious drawback of the manuscript is already acknowledged by the authors themselves (lines 139ff) namely the complete lack of information on whether the antibodies analysed, esp. those produced by the ASC in the late phase of convalescence, are protective? While it would be mandatory to show that any of them are protective, it would be even more informative to learn how much of a viral challenge they can fend off. No data are provided on neutralizing capacity of the sera, not even RBD-binding, nor on secretory IgA, which would qualify to protect mucosal surfaces.

*We agree with the Reviewer that duration of protective antibody responses is a critical point. We analyzed the neutralizing capacity of sera from 25 participants at 1m and 7m post symptom onset against authentic SARS-CoV-2, shown below in Reviewer Fig. 4. At 1m, 24 had neutralizing titers above background against the predominantly circulating strain in the United States carrying the D614G mutation, and at 7m neutralization titers declined in most participants and 16 were neutralizing (panel **a**,* left*). Neutralization capacity was measured against the B.1.1.7 and B.1.351 variants for these 16 samples. While neutralization titers were similar against the B.1.1.7 variant, they were markedly lower against the B.1.351 variant (panel **a**,* center & right*). As many others have demonstrated, the neutralizing titers correlate well with anti-S IgG titers (panel **b**). Because the 1m neutralization data have already been published elsewhere* (Chen et al., 2021) *we have elected to not include them in this manuscript. However, we have added binding titers at 7 and 11m post symptom onset against the receptor binding domain (RBD) of spike, which is a primary target of neutralizing antibodies (Fig. 2d, shown below in panel **c**).*
*While we believe that the vast majority of anti-spike antibodies in serum 7 and 11m post symptom onset are derived from long-lived BMPCs, direct demonstration of the protective capacity of the S-specific plasma cells would require expressing the corresponding monoclonal antibodies from*

*those cells and assessing their neutralizing potential. Apart from being outside the scope of this manuscript, technically this is very challenging task because of the extremely low frequency of these cells, and the fact that we cannot single cell sort these cells using labeled S probes due to lack of surface Ig, a challenge also noted by Reviewer 2.*

[Panels a and b are redacted]




**Reviewer Figure 4. Neutralizing capacity of convalescent serum**

**a,** Neutralization titers of sera collected 1- and 7-months post symptom onset against authentic Washington strain SARS-CoV-2 with the D614G mutation (*left*), the B.1.1.7 variant (*center*), and the B.1.351 variant (*right*). **b,** Serum D614G neutralization titers plotted against respective IgG S-binding titers from convalescent participants 1- and 7-months post symptom onset. *P*- and *r*-values from two-sided Spearman's correlation. Each symbol represents one sample (*n*=25). **c,** Paired anti-RBD IgG serum antibody titers from convalescent participants 7m and 11m post symptom onset. Each symbol represents one sample (*n*=5).

10) Finally, when it comes to the characterization of the circulating SARS-CoV-2-specific memory B cells and their relationship to the ASC, the data fall short of state-of-the-art options to compare their antigen-receptor repertoires, not to speak of transcriptomes. Thus the manuscript remains a bit cryptic and does not add much to our present knowledge that SARS-CoV-2 triggers in many humans a T cell-dependent immune response, including the cognate activation of B cells, and their differentiation into memory B cells and antibody-secreting cells. Protective memory is not demonstrated, and the titers of serum antibodies do fade over the period of observation in most patients over the time of observation, here 7 months. And this has been demonstrated before.

*Comparing S-specific antigen-receptor B cell repertoires between circulating memory B cells and bone marrow plasma cells is an excellent point that also was raised by Reviewer 2 below. However, and as acknowledged by Reviewer 2, this is technically challenging because bone marrow plasma cells cannot be isolated based on antigen binding. Our main goal from the memory B cell figure is to show that we also detect a circulating S-specific resting memory B cellcompartment. We agree with the Reviewer that this point has been reported by multiple groups already, and it is not the main focus of our study.*

**Reviewer #2:**

In this important paper, the persistent production of antibody to SARS-CoV-2 in patients after infection is comprehensively evaluated for up to 8-months at both the serum and, strikingly, at the cellular level in the form of long-lived bone marrow plasma cells. This work evaluates a critical and persistent question: how long will immune protection be maintained after COVID-19 exposure? An important observation for seasonal human coronaviruses is that antibody-mediatedimmunity wanes over time leaving people susceptible to re-infection with the same hCoV strains.While the current literature provides some insight into this issue at the serological level, the

relatively short time span since the outbreak of COVID-19 leads to limited longitudinal sampling such that one can never be certain if antibody present is from peripheral, short-lived sources such as circulating or mucosal-associated plasmablasts. Thus, these observations must always be weighted with the potential that antibody may yet still wane with more time. In this paper they recruited recovered patients and demonstrate that long-lived serum responses do indeed correlate well the presence of apparent long-lived plasma cells in the bone marrow after assessing bone marrow aspirates from 18 patients. It was verified from a control, never-infected cohort thatthe assay was specific and not detecting cross-reactive epitopes from endemic hCoVs, for example.

*We appreciate the supportive summary of the work.*

While the story is convincing as is, more data ensuring that these cells are long-lived plasma cells and not recirculating plasmablasts that were in the bone marrow would be of value, such as flow cytometry verifying that the cells have terminally differentiated and so have no surface Ig, for example.

*We thank the Reviewer for highlighting this important point, which was also raised in Reviewer 1's point #5 (above). As described above, we used preserved bone marrow specimens from the SARS-CoV-2 convalescent patients to examine the expression of proliferation and differentiation markers in spike-specific plasma cells in comparison to bone marrow plasma cells that are specificto influenza virus HA and recently generated spike-specific circulating plasmablasts. Phenotypically, S+ (and influenza virus HA+) bone marrow plasma cells are quiescent as indicated by lack of Ki67 expression, in contrast to recently generated S+ blood plasmablasts. Inaddition, the bone marrow plasma cell populations expressed similar levels of Blimp1 and higherlevels of CD38 compared to circulating plasmablasts.*

Observations are also provided regarding concordant memory B cell frequencies. It would also be of value to link the bone marrow plasma cell Ig repertoire to B cell memory. To clarify, while itis completely understandable that these evaluations may be impossible to obtain based on the limited sampling and the fact that bone marrow plasma cells cannot be isolated based on antigenbinding (they are cell surface Ig-negative), this data would certainly be of great interest.

*We agree with the Reviewer regarding how valuable yet technically challenging such an analysis would be (also discussed in our response to Reviewer 1 #9).*

That said, in my opinion this is an extremely important observation to share and so should be published as soon as possible without delay if additional data is not already available.

*We greatly appreciate the supportive comments about the importance and impact of the study.*

**Reviewer #3:**

In this manuscript, Turner et al identify long-lived human bone marrow plasma cells that are specific for the S protein of SARS-CoV-2. These cells can be detected for at least 8 months afterinfection and their levels correlate with levels of anti-S antibody. The study is important and helpsallay fears that SARS-CoV-2 does not induce a durable antibody response. The limitations of thestudy are described in good detail.

*We appreciate the supportive summary of the work.*

Specific comments.

There was substantial variability in the number of SARS-CoV-2 S protein-specific BMPCs identified in the 18 patients, and these cells could not be detected in 4/18 patients. Although thisis a small number of patients, are there any clinical or laboratory characteristics of the patients that could explain the variability of the response?

*We agree with the Reviewer that this is an intriguing point. We have examined multiple parameters, including age, sex, co-morbidities, concurrent medications, severity and duration of symptoms, and the time span between onset of symptoms and collection of the bone marrow aspirate and could not find a trend that would explain the lack of detectable bone marrow plasma cells in those four participants compared to the rest. As noted by the Reviewer, this could be justan assay limit of detection issue, as we propose in the discussion.*

Which of the patients described in Extended Data Table 1 underwent BM biopsy?

*We thank the Reviewer for this suggestion. Columns in Extended Data Tables 1 & 2 have been added with the demographic and symptom information for the bone marrow cohort.*

**References**

Chen, R.E., Zhang, X., Case, J.B., Winkler, E.S., Liu, Y., VanBlargan, L.A., Liu, J., Errico, J.M., Xie, X., Suryadevara, N., et al. (2021). Resistance of SARS-CoV-2 variants to neutralization by monoclonal and serum-derived polyclonal antibodies. Nat. Med. 10.1038/s41591-021-01294-w.

**Reviewer Reports on the Second Revision:**

Referees' comments:

Referee #1 (Remarks to the Author):

In this revised version of their manuscript, the authors have drastically improved the quality of their work, showing that SARS-CoV-2 generates longlasting humoral immunological memory. The major claim that in immune responses to SARS-CoV-2 longlived memory plasma cells are generated, secreting neutralizing antibodies, is now solidly underpinned, and flanked by convincing data on anti-SRAS-CoV-2 serum antibody dynamics and persistency of memory B cells. Also discussion and citations now are in good shape. All the criticisms on the initial version have been appropriately dealt with.

Referee #2 (Remarks to the Author):

All concerns have been suitably addressed

Referee #3 (Remarks to the Author):

In this revised manuscript, Turner et al present additional data supporting the conclusions that long-lived BM plasma cells are detected in most COVID-19 survivors. The studies are extended to samples obtained 11 months after infection, providing additional evidence of longevity of the response. Additional characterization of the responding cells is provided. At this point, there are a

few minor issues that should be considered.

1. Were any of the subjects vaccinated? They were presumably not, but this should be explicitly mentioned.
2. Extended Data Table 2-The percentages under Total at Month 11 are incorrect. The denominator should be 42.
3. Plasmablast gating should be indicated in Extended Data Figure 1d as in the Letter of Rebuttal.
4. Line 150-"Importantly,…"-These data are discussed in the Letter of Rebuttal but do not seem to be present in the final text. They should be added to one of the figures.

**Author Rebuttals to Second Revision:**

We would again like to thank the editor and reviewers for their time and continued consideration of our manuscript. The comments provided have helped to clarify some important points.

Below we have provided our responses (in *blue*) to Reviewer 3's comments detailing the ways in which our revised manuscript addresses their concerns.

**Reviewer #1:**
In this revised version of their manuscript, the authors have drastically improved the quality of their work, showing that SARS-CoV-2 generates longlasting humoral immunological memory. The major claim that in immune responses to SARS-CoV-2 longlived plasma cells are generated, secreting neutralizing antibodies, is now solidly underpinned, and flanked by convincing data on anti-SARS-CoV-2 serum antibody dynamics and persistency of memory B cells. Also discussion and citations now are in good shape. All the criticisms on the initial version have been appropriately dealt with.

**Reviewer #2:**
All concerns have been suitably addressed.

**Reviewer #3:**
In this revised manuscript, Turner et al present additional data supporting the conclusions that long-lived BM plasma cells are detected in most COVID-19 survivors. The studies are extended to samples obtained 11 months after infection, providing additional evidence of longevity of the response. Additional characterization of the responding cells is provided. At this point, there are a few minor issues that should be considered.

1. Were any of the subjects vaccinated? They were presumably not, but this should be explicitly mentioned.
*This is an important point; we have highlighted this in the results (line 71).*

2. Extended Data Table 2-The percentages under Total at Month 11 are incorrect. The denominator should be 42.
*We thank the Reviewer for bringing this to our attention; the percentages have been corrected.*

3. Plasmablast gating should be indicated in Extended Data Figure 1d as in the Letter of Rebuttal.
*We have added PBMC plasmablast gating to Extended Data Fig. 1.*

4. Line 150-"Importantly,…"-These data are discussed in the Letter of Rebuttal but do not seem to be present in the final text. They should be added to one of the figures.
*We have added this point to the results (line 107). As all PBMC data are negative, we feel it would not be informative to present a plot showing only zero-values.*