

Peer Review File

Manuscript Title: SARS-CoV-2 specific T cell immunity in COVID-19, SARS and uninfected

Editorial Notes:**Redactions – Third Party Material**

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

Reviewer Comments & Author Rebuttals**Reviewer Reports on the Initial Version:**(Referee #1):

The report by Bertoletti and colleagues provides analysis of T cell responses by three groups of subjects.

1. SARS-CoV2 recovered
2. SARS-CoV1 recovered
3. SARS-CoV1/2 unexposed

The whole paper focuses on T cell responses to 3 coronavirus proteins, NP, NSP7 and NSP13.

These are chosen for their homology with SARS-CoV1, and the 4 known “common cold” coronaviruses. Homology is almost 100% with SARS-CoV-1, and less so for the other viruses.

The paper has several interesting findings. First, they show that reactivity by SARS-CoV-2 recovered subjects is strongly biased to SARS-CoV-2 NP, with poor reactivity to the NSPs. They then provide novel data showing that NP reactivity consists of responses to multiple regions of this molecule in most subjects. Importantly, they identify some of the recognised sequences and show some overlap with epitopes previously identified for SARS-CoV-1 immune subjects.

Next, they show that SARS-CoV-1 recovered subjects still have T cell responses to SARS-CoV-1 proteins 17 years after infection, implicating long-lived T cell immunity. The SARS-CoV-1 exposed subjects are then shown to respond to SARS-CoV2 NP, though with slightly lower reactivity. Cross-reactivity is not that surprising here since the proteins chosen are highly homologous. Still, this does demonstrate that there are cross-reactive T cells that can respond to SARS-CoV-2.

Interestingly, their analysis of responses by unexposed subjects shows a quite different reactivity pattern, with greater focus on the non-structural proteins NSP7/13 over NP and a bias to the NP1 pool of peptides. This pattern was seen in an earlier report (ref 11), but not as clearly documented. Bertoletti's group further show that these responses can be amplified by culture, implying they might be expanded if subjects were infected with SARS-CoV-2. For me, this finding raises the question as to why cross-reactive NSP responses do not dominate the responses of SARS-CoV-1 or SARS-CoV-2 recovered subjects in Fig 1 and Fig 3, respectively, since, in theory, about half of these subjects should have had cross-reactive T cells prior to infection (based on unexposed subjects). Some comment here would be useful.

To extend the analysis of cross-reactivity further, the authors identify the peptide sequences responsible for stimulation in some unexposed subjects. This novel data provides evidence that some epitopes may be derived from cross-reactive responses to “common cold” coronaviruses. The more interesting finding, however, is that one immunogenic CD8 epitope is not well conserved in these “common cold” coronaviruses, raising the important question of how this response was originally generated. The authors speculate that it may be through cross-reactive stimulating by non-infectious animal coronaviruses. This hypothesis is very interesting but highly speculative. One experiment that might be achievable quickly, is to synthesise all the homologous peptides from the “common cold” coronaviruses and show that they do not stimulate this T cell response (as implied). It might be that conservation, while poor, is sufficient to be stimulatory, despite

amino acid changes. It would also be interesting to synthesise and test similar regions from common animal (cat?, dog?, mice?) coronaviruses, if sequences are known. Overall, I found this paper highly topical and thought provoking.

I have a number of minor concerns:

General minor concern: how can we know what the true naïve response is? In other words, it is implied/claimed that the unexposed subjects have cross-reactive responses, but how do we know that they are not just low affinity naïve responses to peptides. Afterall, most of the responses are pretty small. Why aren't these responses seen in the exposed subject (where do these responses go), who show little reactivity to NSPs? Some comment in the discussion would be helpful.

Figure 1. the legend and figure do not match the main text with respect to numbering. The main text seems to have been written before Figure 1D existed. In addition, the actual Figures in Figure 1C and D are never referred to in the main text.

Figure 1E. No examples of CD4 staining are shown. The Methods section indicates CD4 staining was used in ICS. It would be good to show some examples here too, otherwise the reader is led to believe CD4 T cell numbers were implied from the CD8-negative cells, which I assume was not the case.

Figure 1D vs E. Some of the numbers do not seem to add up, though it may just be variation between assays. For example, C15 responds equally in elispot to NP1 and NP2 pools, but only shows ICS IFNg for CD4 T cells to NP1 pool. The data in 1E (right graphs with circle symbols) is also very hard to interpret. What is the background? Can the symbols also have some different shapes for colors that are close e.g. C6 and C15, AND, C22 and C9.

Figure 2A. For clarity, can you mark which samples were from direct ex vivo and which were from expansion. Can you please clarify which peptides were used in the in vitro expansion? What are the * symbols for in Fig 2A, next to some of the subjects' IDs?

Figure 2B. Example dot-plots to the right of the table show primarily CD8-negative TNF+ T cell responses. Methods say you used CD4 staining, so why not show this? Why are there no examples of CD8 responses here?

Figure 4. In Fig 4A some donors are pre-July 2019 and others are sero-negative for SARS-CoV2 neutralizing Ab and NP Ab. Can these two groups be distinguished by symbol shape, so that we know if there are any differences there?

I think figure 4B would be slightly more informative if the two circles could be merged to also show those patients that responded to both NP and NSPs, with subjects having both responses being co-colored dark grey and green hashed. Otherwise, we cannot tell the cumulative total of all subjects that responded to these proteins.

There is something slightly missing in the logic of the last 2 sentences of the discussion. It is unclear why the word "nevertheless" is used. I think a step in logic is missing.

(Referee #2)

A:

Le Bert et al investigate T cell responses to SARS-CoV1, -CoV2 structural and non-structural proteins. The authors identify long-lasting T cell memory to SARS-CoV1 that cross-react with SARS-CoV2 peptides and also that unexposed individuals have SARS-CoV2 reactive T-cells. The report confirms recent data on the development of T-cell memory responses in SARS-CoV2, it highlights immunodominance patterns of T-cell memory. The finding that unexposed individuals have SARS-Cov2 reactive T-cells is not surprising since most healthy individuals have memory T-

cells to a range of pathogens not encountered as a consequence of T-cell cross-reactivity (Su et al, Immunity, 2013).

B: Overall, the paper confirms several expected aspects of T-cell immunity to SARS-CoV2, it validates the assumption that T-cell responses are elicited but it does not address the important issue of a possible link between prior T-cell immunity and reactivity and disease severity in COVID-19.

Another critical issue in COVID-19 relates to the quality of the elicited B-cell response and the development of neutralizing antibodies. A significant proportion of symptomatic patients with COVID-19 fail to develop neutralizing antibodies (Robbiani et al, BioRxiv 2020), leading to the possibility of antibody-mediated enhancement as a possible mechanism of prolonged or worsened COVID-19 in subsets of patients seen after about 7-10 days. The authors could add important information by linking the quality of the T-cell response, prior T-cell immunity and its specificity with the development of neutralizing antibodies in these patients.

C,D,E,F:

Detailed suggestions and questions

- Is there any relationship between reactivity (ELISpot IFN γ SFU) vs viral clearance, i.e time until negative PCR in SARS-CoV-2 patients?
- Acknowledge small sample size.
- In figure 4, the authors show that healthy unexposed control PBMCs produce IFN γ in response to SARS-CoV-2 peptides, indicating underlying memory responses to other coronaviruses. Authors should discuss these findings in more depth in terms of specificity of T cells (findings from figures 1-3).

Figure 1: T cells from recovered COVID-19 patients secrete TNF- α and IFN- γ in response to SARS-CoV-2 peptides, most consistently to NP peptides.

Legend:

- Inconsistency in labelling Figure 1D (as 1C in legend and results section); and of Figure 1E (as 1D in legend and results section). r

Questions/ comments:

- Figure 1 does not display comparison of responses between COVID-19 recovered patients and healthy controls. Although low responses to NSP7 and NSP13 serve as negative biological control, comparing results to healthy uninfected controls would strengthen conclusions as to the specificity of the responses.
- Display gating strategy for Figure 1E (which is probably the same for 2B and 4D)
- Due to low frequency of TNF α /IFN γ -producing T cells, would the authors expect these subjects to display sufficient secondary responses to SARS-COV2?

Figure 2: Identification of multiple regions within NP protein that induce T cell responses in recovered COVID19 patients (IFN γ ELISpot and TNF α intracellular flow cytometry). Some of the identified regions have been also previously shown to be targeted by T cells of SARS recovered subjects.

Questions/ comments:

- Again, none of the panels in this figure show responses in healthy uninfected controls.
- Regarding 2B: Authors should show characterization and frequency of T cells following T cell expansion assay to actually call them "T cells", "memory T cells", etc. If not possible, then text should be rewritten to state what's actually being shown. Same is true for text referring to Figs 3E and 4D.

Figure 3: Individuals that recovered from SARS 17y and are unexposed to COVID19 ago still

produce IFN γ to SARS-CoV-1 peptides (predominantly to NP peptides) and also display IFN γ responses to SARS-CoV-2 NP peptides.

Legend:

- Figures legends for 3D and 3E need to indicate what is being measured (IFN γ SFU?).

Questions/ comments:

- None of the panels in this figure show responses in healthy uninfected controls. Again, although low responses to NSP7 and NSP13 serve as negative biological control, comparing results to healthy uninfected controls would strengthen conclusions.
- Figure 4A is mentioned between figures 3D and 3E
- Authors show PBMC production of IFN γ in response to SARS-CoV-1 peptides in SARS recovered subjects, but don't delineate source or specificity of responses in Figures 3A-D. Lines 150-160 in the text need to be changed to match conclusions to experimental design.

Figure 4: Unexposed healthy donors display IFN γ responses to SARS-Cov-2 peptides (NP and NSP proteins)

Legend:

- 4A) "Frequency of peptide-reactive T cells is shown for each donor (dots) and the bars represent median frequency." The assay shows frequency of peptide-reactive PBMC.
- 4B) "Pie charts represent percentage of individuals with NP-specific and NSP7/13-specific T cells for unexposed, SARS- and COVID-19- recovered individuals." – same as above.
- 4C) "Frequency of SARS-CoV-2 reactive T cells in 9 unexposed donors to the indicated peptide pools directly ex vivo and after a 10-day expansion." – same as above.
- 4D) Clarify in legend whether PBMCs or expanded cells were stimulated with +-peptides.
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Questions/ comments:

- Same as in Figure 3, text (lines 188-200) describes reactivity of T cells to peptides when assay shows reactivity of PBMCs (Figure 4A-C). Change text to match conclusions to experimental design.

Methods

- ELISpot assay: specify how long PBMCs were stimulated for with peptide pools.

G: References credit previous work of importance.

H: The paper is clearly written overall.

(Referee #3)

In this well written manuscript, Le Bert et al measure T cell responses in patients who recovered from SARS and COVID-19 and in those who developed neither disease. The studies concentrated on two nsps, nsp7 and nsp13 and on the nucleocapsid a well-described target for the T cell and antibody response. The N protein is highly conserved when SARS-CoV and SARS-CoV-2 sequences are compared so is useful for demonstrating cross-reactivity. A novel aspect of the manuscript is the demonstration that anti-virus T cell responses can be detected in SARS survivors 17 years after the epidemic ended and that T cell responses also recognized SARS-CoV-2 epitopes. These results are convincing but not very surprising since previous studies showed that SARS-CoV-specific T cells were detected at 11 years post infection. The last part of the manuscript includes data showing that SARS-CoV-2 T cell responses were also detected in healthy donors who had no pre-existing history of SARS or SARS-CoV-2 infection, which if validated might provide information about pre-existing immune protection (or pathogenicity).

Specific comments.

1. T cell responses are measured by ELISPOT and intracellular cytokine staining for IFN-g and TNF expression, which are excellent approaches.

Figure 1E-Most cells are expected to be positive for IFN-g and TNF expression rather than singly positive for one or the other. Staining for IFN-g vs. TNF should be shown in the flow plots and the summary data in the right hand panel changed to reflect this overlap, if confirmed. If cells are mostly singly positive for one or the other cytokine, this should be discussed.

2. Given the unexpected findings described in Figure 4, the data should be reinforced by additional supporting quantitative assays. In addition to ELISPOT and in vitro stimulation data, direct ex vivo peptide stimulation assays should be shown, measuring IFN-g and TNF single and double positives as described above. The data in Figure 4D may already show direct ex vivo staining, but this is not clear. Detection of double cytokine producing cells, especially in the case of nsp-specific T cell responses, will show that such cells are present in more than tiny numbers.

-Figure 4D, subject H-2-Was an anti-N antibody response detected in this patient, give the robust N-specific T cell response?

-Line 216-unless the T cell responses in HD are shown to be more robust, it is premature to draw any conclusions about their origin. The lack of obvious congruency of nsp sequences with common cold nsp CoV sequences is notable, especially since this contrasts with results obtained from studies of N-specific peptides, which are shared among the CoV. It is equally possible that these putative cross-reacting T cells were primed by an unrelated virus or other pathogen rather than by a novel CoV.

3. Line 238-Coronaviral proteins are all produced in the same ratio throughout infection. There is no evidence for temporal differences in expression.

Author Rebuttals to Initial Comments:

Point-by-point response

Referee #1 (Remarks to the Author):

The report by Bertoletti and colleagues provides analysis of T cell responses by three groups of subjects.

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2. SARS-CoV1 recovered
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These are chosen for their homology with SARS-CoV1, and the 4 known "common cold" coronaviruses. Homology is almost 100% with SARS-CoV-1, and less so for the other viruses.

The paper has several interesting findings. First, they show that reactivity by SARS-CoV-2 recovered subjects is strongly biased to SARS-CoV-2 NP, with poor reactivity to the NSPs. They then provide novel data showing that NP reactivity consists of responses to multiple regions of this molecule in most subjects. Importantly, they identify some of the recognised sequences and show some overlap with epitopes previously identified for SARS-CoV-1 immune subjects.

Next, they show that SARS-CoV-1 recovered subjects still have T cell responses to SARS-CoV-1 proteins 17 years after infection, implicating long-lived T cell immunity. The SARS-CoV-1 exposed subjects are then shown to respond to SARS-CoV2 NP, though with slightly lower reactivity. Cross-reactivity is not that surprising here since the proteins chosen are highly homologous. Still, this does demonstrate that there are cross-reactive T cells that can respond to SARS-CoV-2.

Interestingly, their analysis of responses by unexposed subjects shows a quite different reactivity pattern, with greater focus on the non-structural proteins NSP7/13 over NP and a bias to the NP1 pool of peptides. This pattern was seen in an earlier report (ref 11), but not as clearly documented.

Bertoletti's group further show that these responses can be amplified by culture, implying they might be expanded if subjects were infected with SARS-CoV-2. For me, this finding raises the question as to why cross-reactive NSP responses do not dominate the responses of SARS-CoV-1 or SARS-CoV-

2 recovered subjects in Fig 1 and Fig 3, respectively, since, in theory, about half of these subjects should have had cross-reactive T cells prior to infection (based on unexposed subjects). Some comment here would be useful.

Answer #1.1 *We agree with this comment. NSP-responses remain minimal in subjects who experienced a SARS-CoV-1 and SARS-CoV-2 infection, even though the analysis of additional recovered COVID-19 subjects added in this second version showed that NSP-responses can be detected in 12 out of 36 subjects tested. However, they are never the dominant response (see Fig 1c, d, 3b, d and Extended Data Fig 7). The reason for this difference remains speculative. Our hypothesis, as now clarified in the Discussion, is that the dominance of structural protein specific responses detected in SARS-CoV-1 and SARS-CoV-2 infected subjects is a consequence of the large production of structural viral proteins (particularly NP) in infected cells in comparison to ORF-1 gene products (see Irigoyen N, et al. High-Resolution Analysis of Coronavirus Gene Expression by RNA Sequencing and Ribosome Profiling. PLoS Pathog. 2016;12(2):e1005473). Dominance of NSP-specific T cell responses in some “uninfected subjects” might be caused by the fact that they are “exposed but uninfected” individuals. As we discussed in page 12 (see also answer #3.5), coronaviridae first translate the replicase gene (ORF1) upon infection. The NSP proteins coded by ORF-1 (among them NSP-7 and 13) form the replicase-transcriptase complex responsible for the transcription of the sub-genomic RNA and subsequent production of viral structural proteins. This raises the possibility that these NSP-specific T cells have been primed by an abortive initial infection with coronaviruses, and it is supported by the observation that NSP-7 T cells in “uninfected individuals” have a classical memory/ TEMRA phenotype (see new data in Extended Data Fig. 10). We have discussed this hypothesis in the conclusions.*

To extend the analysis of cross-reactivity further, the authors identify the peptide sequences responsible for stimulation in some unexposed subjects. This novel data provides evidence that some epitopes may be derived from cross-reactive responses to “common cold” coronaviruses. The more interesting finding, however, is that one immunogenic CD8 epitope is not well conserved in these “common cold” coronaviruses, raising the important question of how this response was originally generated. The authors speculate that it may be through cross-reactive stimulating by non-infectious animal coronaviruses. This hypothesis is very interesting but highly speculative. One experiment that might be achievable quickly, is to synthesise all the homologous peptides from the “common cold” coronaviruses and show that they do not stimulate this T cell response (as implied). It might be that conservation, while poor, is sufficient to be stimulatory, despite amino acid changes. It would also be interesting to synthesise and test similar regions from common animal (cat?, dog?, mice?) coronaviruses, if sequences are known.

Answer #1.2. *We thank the reviewer for this suggestion. We synthesized the homologous peptides from the “common cold” coronaviruses that correspond to this region of NSP7. We now directly show that these peptides do not stimulate SARS-CoV-2 NSP-7-specific responses (directly in PBMC of the two unexposed subjects with NSP7 36-50 specific T cells and in a NSP7 36-50-specific short-term T cell line). These new results are shown in Extended Data Fig. 9b.*

Overall, I found this paper highly topical and thought provoking.

I have a number of minor concerns:

General minor concern: how can we know what the true naïve response is? In other words, it is implied/claimed that the unexposed subjects have cross-reactive responses, but how do we know that they are not just low affinity naïve responses to peptides. Afterall, most of the responses are pretty small. Why aren't these responses seen in the exposed subject (where do these responses go), who show little reactivity to NSPs? Some comment in the discussion would be helpful.

Answer #1.3. *We extended our discussion, see Answer #1.1. Moreover, the reviewer is correct in pointing out that we cannot completely exclude that in some uninfected subjects the NP and NSP-specific responses are sustained by naïve T cells, which we now discuss in line 237. However, in two uninfected donors with NSP-7-specific T cells, we demonstrate that they are of memory and TEMRA phenotype (Extended data Fig. 10).*

Figure 1. the legend and figure do not match the main text with respect to numbering. The main text seems to have been written before Figure 1D existed. In addition, the actual Figures in Figure 1C and D are never referred to in the main text.

Answer #1.4. *We corrected these inaccuracies.*

Figure 1E. No examples of CD4 staining are shown. The Methods section indicates CD4 staining was used in ICS. It would be good to show some examples here too, otherwise the reader is led to believe CD4 T cell numbers were implied from the CD8-negative cells, which I assume was not the case.

Answer #1.5. *We added examples of CD4 staining as requested.*

Figure 1D vs E. Some of the numbers do not seem to add up, though it may just be variation between assays. For example, C15 responds equally in elispot to NP1 and NP2 pools, but only shows ICS IFN γ for CD4 T cells to NP1 pool. The data in 1E (right graphs with circle symbols) is also very hard to interpret. What is the background? Can the symbols also have some different shapes for colors that are close e.g. C6 and C15, AND, C22 and C9.

Answer #1.6. *Yes, in a few subjects the ratio of responses detected with ICS did not perfectly match with the ELISpot pattern. As suggested, instead of colours, we use now different symbols. The background differs in every subject, but examples of ICS background from un-stimulated PBMC are shown in the dot plots. For the summary figure, we subtracted the background to make the figure more readable.*

Figure 2A. For clarity, can you mark which samples were from direct ex vivo and which were from expansion. Can you please clarify which peptides were used in the in vitro expansion? What are the * symbols for in Fig 2A, next to some of the subjects' IDs?

Answer #1.7. *We apologize, asterisked subjects in column 1 of the table in Fig.2a are those in whom responses were measured after in vitro expansion, which we have now clarified in the figure legend. For in vitro expansion, PBMC were stimulated with all three peptide pools combined as described in Methods.*

Figure 2B. Example dot-plots to the right of the table show primarily CD8-negative TNF+ T cell responses. Methods say you used CD4 staining, so why not show this? Why are there no examples of CD8 responses here?

Answer #1.8. *As requested, we now show examples of CD4 and CD8 responses (Fig 2c).*

Figure 4. In Fig 4A some donors are pre-July 2019 and others a sero-negative for SARS-CoV2 neutralizing Ab and NP Ab. Can these two groups be distinguished by symbol shape, so that we know if there are any differences there?

Answer #1.9 *We introduced the requested modifications.*

I think figure 4B would be slightly more informative if the two circles could be merged to also show those patients that responded to both NP and NSPs, with subjects having both responses being co-colored dark grey and green hashed. Otherwise, we cannot tell the cumulative total of all subjects that responded to these proteins.

Answer #1.10 *We thank the reviewer for the suggestion to change Fig. 4b, which we agree, is now more informative.*

There is something slightly missing in the logic of the last 2 sentences of the discussion. It is unclear why the word “nevertheless” is used. I think a step in logic is missing.

Answer #1.11. *Apologies. The authors are not native English speaking. We modified the sentence.*

Referee #2 (Remarks to the Author):

A: Le Bert et al investigate T cell responses to SARS-CoV1, -CoV2 structural and non-structural proteins. The authors identify long-lasting T cell memory to SARS-CoV1 that cross-react with SARS-CoV2 peptides and also that unexposed individuals have SARS-CoV2 reactive T-cells. The report confirms recent data on the development of T-cell memory responses in SARS-CoV2, it highlights immunodominance patterns of T-cell memory. The finding that unexposed individuals have SARS-CoV2 reactive T-cells is not surprising since most healthy individuals have memory T-cells to a range of pathogens not encountered as a consequence of T-cell cross-reactivity (Su et al, Immunity, 2013).

B: Overall, the paper confirms several expected aspects of T-cell immunity to SARS-CoV2, it validates the assumption that T-cell responses are elicited but it does not address the important issue of a possible link between prior T-cell immunity and reactivity and disease severity in COVID-19.

Answer #2.1. *The reviewer is correct, we didn't address the role of prior T cell immunity in protection and disease severity of COVID-19, a question that is difficult to address without the use of an animal model or large longitudinal population studies that are outside the scope of this study. This is why we were careful not to claim anywhere in the manuscript that our data demonstrate the protective capacity of such pre-existing T cell immunity. Our work was designed to understand the immunodominance of T cell responses against structural and non-structural proteins in COVID-recovered subjects, the existence/ persistence of a memory SARS-CoV-2 specific T cell response in subjects infected many years ago by SARS-CoV-1 and the possible presence of SARS-CoV-2 specific T cells in unexposed individuals. We think that our data provide the immunological basis to initiate animal studies or large epidemiological studies to understand whether the presence of "prior immunity" might induce protection or alter the disease course.*

Another critical issue in COVID-19 relates to the quality of the elicited B-cell response and the development of neutralizing antibodies. A significant proportion of symptomatic patients with COVID-19 fail to develop neutralizing antibodies (Robbiani et al, BioRxiv 2020), leading to the possibility of antibody-mediated enhancement as a possible mechanism of prolonged or worsened COVID-19 in subsets of patients seen after about 7-10 days. The authors could add important information by linking the quality of the T-cell response, prior T-cell immunity and its specificity with the development of neutralizing antibodies in these patients.

Answer #2.2. *We agree that the analysis of the quality of B cell response and the development of neutralizing antibodies are critical issues in the Covid-19 pandemic. Thus, even though this is not the primary goal of this manuscript, we followed the reviewer's suggestions and measured the quantity of neutralizing antibodies present in the sera of our COVID-19 recovered subjects. We correlated neutralizing antibody levels with the frequency of IFN-gamma spots detected in peptide-stimulated PBMC as requested, but we found no association (see Extended Data Fig 5). A correlation might however still exist with T cell responses to the spike region, as shown in a recent preprint (Dong T, et al. Broad and strong memory CD4+ and CD8+ T cells induced by SARS-CoV-2 in UK convalescent COVID-19 patients. bioRxiv. June 2020:1-36. doi:10.1101/2020.06.05.134551, which we did not examine.*

Interestingly, however the subjects with severe disease have higher level of neutralizing antibodies. In addition, we found that patients with low levels of neutralizing antibodies can have a robust response to NP and NSP peptide pool.

We thank the reviewer for suggesting the inclusion of such data that we will enhance our understanding of the interplay between B- and T-cells in COVID-19. Note, we employed a novel surrogate virus neutralization test (sVTN) (Preprint at Research Square: doi:10.21203/rs.3.rs-24574/v1). Specifically, it measures the quantity of anti-Spike antibodies that block protein-protein interactions between the binding domain of spike (RBD) to the ACE2 receptor using an ELISA-based assay. Various published studies (including the Robbiani et al, BioRxiv 2020 study cited by this reviewer) have shown that the anti-RBD antibodies are by far the most dominant in neutralizing SARS-CoV-2. This is also true for our sVNT data. The neutralizing antibody level determined by sVNT

correlates very well with the quantity of neutralizing antibodies measured with the conventional virus neutralization test (cVNT) using live virus in BSL3 lab or the pseudovirus based VNT (pVNT) in BSL2 lab (see figure below). This test is now commercialised under the trade name cPass and has obtained approval for clinical testing in Singapore and EU.

[redacted]

C,D,E,F:

Detailed suggestions and questions

- Is there any relationship between reactivity (ELISpot IFN γ SFU) vs viral clearance, i.e time until negative PCR in SARS-CoV-2 patients?
- Acknowledge small sample size.

Answer #2.3. *As requested, the relationship between (ELISpot IFN γ SFU) and time until negative PCR in SARS-CoV-2 patients is now shown in the Extended Data Fig. 5. The small samples size for such correlative analyses is acknowledged in line 128.*

- In figure 4, the authors show that healthy unexposed control PBMCs produce IFN γ in response to SARS-CoV-2 peptides, indicating underlying memory responses to other coronaviruses. Authors should discuss these findings in more depth in terms of specificity of T cells (findings from figures 1-3).

Answer #2.4 *We added more data to show the characteristics of NSP-specific T cells present in uninfected subjects (frequency directly ex vivo, ability to expand, phenotype and HLA-restriction) and we extended the discussion of their specificity in the studied cohorts, see also Answer #1.1 (Reviewer 1).*

Figure 1: T cells from recovered COVID-19 patients secrete TNF- α and IFN- γ in response to SARS-CoV-2 peptides, most consistently to NP peptides.

Legend:

- Inconsistency in labelling Figure 1D (as 1C in legend and results section); and of Figure 1E (as 1D in legend and results section). r

Answer #2.5. *We corrected these inaccuracies.*

Questions/ comments:

- Figure 1 does not display comparison of responses between COVID-19 recovered patients and healthy controls. Although low responses to NSP7 and NSP13 serve as negative biological control, comparing results to healthy uninfected controls would strengthen conclusions as to the specificity of the responses.

Answer #2.6. *We have shown the results of ELISpots of 37 healthy controls in Fig. 4, where we display the comparison of responses detected in the three cohorts tested (Fig 4a). We decided to*

organize our data first reporting the responses obtained in subjects who recovered from COVID-19 and SARS (Fig. 1c and Fig. 3b). We then compared these results with the ones obtained in the unexposed individuals (Fig. 4a). The addition of healthy control results in Fig. 1 would require a complete reorganization of the flow of the manuscript, and we don't think it will increase clarity. However, we agree with the reviewer that an additional figure showing direct comparison of the different responses in these different cohorts would be helpful. Thus, in addition to Fig. 4a (showing the quantity of spots detected against the different peptides pools in all three cohorts), we added Extended Data Fig. 7, where the composition of reactive cells against NP1, NP2, NSP7 and 13 present in the individual subjects of the three cohorts are compared.

Furthermore, the specificity of an ELISpot assay is not primarily derived from comparison with results obtained in "healthy controls" but on comparison with responses from unstimulated PBMC. Subsequently, a confirmation assay using intracellular cytokine staining is applied, to show that peptide pools stimulate T cells (CD4 or CD8) to produce IFN-gamma and TNF-alpha. Our data showing responses in uninfected controls serve to underscore why they cannot be used as specificity controls for the assay.

Display gating strategy for Figure 1E (which is probably the same for 2B and 4D)

Answer #2.7. The gating strategy is now displayed in Extended Data Fig. 3.

- Due to low frequency of TNFa/IFNg-producing T cells, would the authors expect these subjects to display sufficient secondary responses to SARS-CoV2?

Answer #2.8 The frequency of these T cells is compatible with the frequency of memory T cells present in other viral infections.

For example, we would not define the frequency of SARS-CoV-2 specific T cells as low if we compare them with HBV-specific T cells detectable in patients who resolved acute HBV infection (see for example Webster GJM, et al. Longitudinal Analysis of CD8+ T Cells Specific for Structural and Nonstructural Hepatitis B Virus Proteins in Patients with Chronic Hepatitis B: Implications for Immunotherapy. *Journal of Virology*. 2004;78(11):5707-5719. Tan AT, Loggi E, Boni C, et al. Host ethnicity and virus genotype shape the hepatitis B virus-specific T-cell repertoire. *Journal of Virology*. 2008;82(22):10986-10997).

In addition, we show that these SARS-CoV-2 T cells are able to efficiently expand in vitro, suggesting they are capable of initiating robust clonal expansion following a new viral challenge. Thus, we postulate that these T cells could mount a robust secondary response. Whether such a response is sufficient for protection from re-infection, is a question that we cannot answer with this study design. However, we agree that the frequency of SARS-CoV-2-NP-specific T cells is not equivalent to what is detected for T cells specific for HCMV and EBV. Thus we deleted the definition "strong T cell-immunity" that was present in the abstract.

Figure 2: Identification of multiple regions within NP protein that induce T cell responses in recovered

COVID19 patients (IFN γ ELISpot and TNF α intracellular flow cytometry). Some of the identified regions have been also previously shown to be targeted by T cells of SARS recovered subjects.

Questions/ comments:

- Again, none of the panels in this figure show responses in healthy uninfected controls.

Answer #2.9 See answer #2.6.

- Regarding 2B: Authors should show characterization and frequency of T cells following T cell expansion assay to actually call them "T cells", "memory T cells", etc. If not possible, then text should be rewritten to state what's actually being shown. Same is true for text referring to Figs 3E and 4D.

Answer #2.10. *We are not sure that we fully understand this comment. We are showing in Fig. 2b and 2c the characterization of some short-term cell lines generated with peptides mixtures. These cells were expanded in vitro and the short-term cell lines were analyzed with flow cytometry-based ICS assay. The cells present in these short-term cell lines are CD3+ cells and we showed that the cells responsive to individual peptides were producing classical T cell cytokines (i.e. IFN-gamma) and express CD4 or CD8 molecules. Based on these results, we think we can clearly call these responsive cells, T cells.*

Figure 3: Individuals that recovered from SARS 17y and are unexposed to COVID19 ago still produce IFN γ to SARS-CoV-1 peptides (predominantly to NP peptides) and also display IFN γ responses to SARS-CoV-2 NP peptides.

Legend:

- Figures legends for 3D and 3E need to indicate what is being measured (IFN γ SFU?).

Answer #2.10. *We added this information now directly on the axis of the figures*

Questions/ comments:

- None of the panels in this figure show responses in healthy uninfected controls. Again, although low responses to NSP7 and NSP13 serve as negative biological control, comparing results to healthy uninfected controls would strengthen conclusions.

Answer #2.11 See answer #2.6

- Figure 4A is mentioned between figures 3D and 3E.

Answer #2.12 *We corrected this inaccuracy by reorganising the results of Fig. 3.*

- Authors show PBMC production of IFN γ in response to SARS-CoV-1 peptides in SARS recovered

subjects, but don't delineate source or specificity of responses in Figures 3A-D. Lines 150-160 in the text need to be changed to match conclusions to experimental design.

Answer #2.13. *We apologize for this inaccuracy. Reviewer 2 is correct to point out that the text did not match the experimental design. Hence, we changed the text, we quote specific references and we show in Extended Data Fig. 6 the direct demonstration that peptide responsive cells of SARS patients were T cells.*

Figure 4: Unexposed healthy donors display IFN γ responses to SARS-Cov-2 peptides (NP and NSP proteins)

Legend:

- 4A) "Frequency of peptide-reactive T cells is shown for each donor (dots) and the bars represent median frequency." The assay shows frequency of peptide-reactive PBMC.
- 4B) "Pie charts represent percentage of individuals with NP-specific and NSP7/13-specific T cells for unexposed, SARS- and COVID-19- recovered individuals." – same as above.
- 4C) "Frequency of SARS-CoV-2 reactive T cells in 9 unexposed donors to the indicated peptide pools directly ex vivo and after a 10-day expansion." – same as above.

Answer #2.13. *We corrected the legends.*

- 4D) Clarify in legend whether PBMCs or expanded cells were stimulated with +-peptides.

Answer #2.14 *We now added the requested information directly to the Fig 4e (previously 4d).*

Questions/ comments:

- Same as in Figure 3, text (lines 188-200) describes reactivity of T cells to peptides when assay shows reactivity of PBMCs (Figure 4A-C). Change text to match conclusions to experimental design.

Answer #2.15. *The text was changed to match the experimental design.*

Methods

- ELISpot assay: specify how long PBMCs were stimulated for with peptide pools.

Answer #2.16. *ELISpot were performed for 18 hours in the presence of peptides. We added the requested information.*

G: References credit previous work of importance.

H: The paper is clearly written overall.

Referee #3 (Remarks to the Author):

Specific comments.

1. T cell responses are measured by ELISPOT and intracellular cytokine staining for IFN-g and TNF expression, which are excellent approaches.

Figure 1E-Most cells are expected to be positive for IFN-g and TNF expression rather than singly positive for one or the other. Staining for IFN-g vs. TNF should be shown in the flow plots and the summary data in the right hand panel changed to reflect this overlap, if confirmed. If cells are mostly singly positive for one or the other cytokine, this should be discussed.

Answer #3.1. *We provided the requested data in Extended Data Fig. 4. However, since the functional characterization of cytokine production by SARS-CoV-2 specific T cells does not constitute the main focus of our work and since objectively, the quantity of T cells analyzed by ICS directly ex vivo is limited, we prefer not to extensively discuss these data in the main text.*

2. Given the unexpected findings described in Figure 4, the data should be reinforced by additional supporting quantitative assays. In addition to ELISPOT and in vitro stimulation data, direct ex vivo peptide stimulation assays should be shown, measuring IFN-g and TNF single and double positives as described above. The data in Figure 4D may already show direct ex vivo staining, but this is not clear. Detection of double cytokine producing cells, especially in the case of nsp-specific T cell responses, will show that such cells are present in more than tiny numbers.

Answer #3.2. *The dot plots reported in Fig 4d showing responses against NSP peptides that lack homology with “common cold” coronaviruses were derived from an ex vivo analysis of IFN-gamma and TNF-alpha producing T cells. We apologize for not providing the correct information in Fig. 4d. We have now modified the figure labelling and legends, and the figure shows with clarity that a sizeable quantity of NSP-specific T cells was detected ex vivo.*

Furthermore, we agree that “the unexpected findings described in Figure 4” should be reinforced by additional data. Thus, we added new data and new figures (Extended Data Fig. 9 and 10) where we report results that characterize in more detail some of the NSP-T cell responses detected with ex vivo and in vitro ELISPOT and ICS assays.

In particular we provide:

- a) The inability of these SARS-CoV-2 NSP-7 specific T cells to recognize the homologous sequence present in “common cold” coronaviruses (Extended Data Fig. 9b).*
- b) Ex vivo characterization of NSP7-specific T cells producing IFN-gamma and TNF-alpha in a new unexposed donor (H-21).*
- c) The frequency of IFN-gamma/TNF-alpha producing T cells present ex vivo in the 3 unexposed subjects with T cells specific for the NSP7 peptides (Extended Data Fig. 10a).*

- d) *The demonstration of the ability of NSP7-specific T cells to expand in vitro (Extended Data Fig. 10a).*
- e) *The HLA-class I restriction of the CD8 T cells specific for NSP7 aa region 36-40 (Extended Data Fig. 10b).*
- f) *The phenotype of NSP7-specific CD8 T cells of two unexposed individuals (Extended Data Fig. 10c).*

In addition, we were able to trace PBMC collected at different time points before autumn 2019 of subject H-2, who presents a CD4+ T cell response against NP-101-120 of SARS-CoV-2 (Fig. 4e- left panel) and we demonstrated the presence of such T cell reactivity over time (Extended Data Fig. 8).

We think that all this information confirms the existence of a robust SARS-Cov2 cross-reactive T cell response in SARS-Cov1/2-unexposed subjects.

-Figure 4D, subject H-2-was an anti-N antibody response detected in this patient, given the robust N-specific T cell response?

Answer #3.3. *No, this subject was negative for anti-NP-antibodies. Thus, we could not find a correlation between presence of NP-specific T cells and anti-NP antibodies.*

-Line 216-unless the T cell responses in HD are shown to be more robust, it is premature to draw any conclusions about their origin. The lack of obvious congruency of nsp sequences with common cold nsp CoV sequences is notable, especially since this contrasts with results obtained from studies of N-specific peptides, which are shared among the CoV. It is equally possible that these putative cross-reacting T cells were primed by an unrelated virus or other pathogen rather than by a novel CoV.

Answer #3.4. *We thank the Reviewer for this comment. We agree and we have introduced in the text the notion that other micobes/pathogens can induce cross-reactive T cells (see line 237).*

3. Line 238-Coronaviral proteins are all produced in the same ratio throughout infection. There is no evidence for temporal differences in expression.

Answer #3.5. *We agree that during established infection, all coronavirus proteins may be produced at a comparable ratio. Our, hypothesis, that ORF-1-specific T cells might recognize infected cells that are not yet producing a large quantity of virions, is derived from the description of the unusual transcription strategy of coronaviruses in many studies. In all coronaviruses, after receptor binding and fusion, the next step is the translation of the replicase gene (ORF1). The replicase-transcriptase complex is then responsible for creating an environment permissive to the transcription of the sub-genomic RNA.*

For example, in the review “Coronaviruses post-SARS: update on replication and pathogenesis” Nature Review Microbiology volume 7, page 439-450 (2009) Stanley Perlman wrote: “Following entry into the cell and uncoating, the positive sense RNA genome is translated to generate replicase proteins from open reading frame 1a/b (ORF1a/b). These proteins use the genome as a template to generate full-length negative sense RNAs, which subsequently serve as templates in generating additional full-length genomes. “

We think this supports the validity of our hypothesis that “an ORF-1-specific T cell can be envisaged to abort viral production in infected cells by lysis of SARS-CoV-2 infected cells.”

However, since we realized that this concept was not clear and that such possibility is only a hypothesis and not directly experimentally proven, we modified the sentence as follows:

:Theoretically, individuals exposed to coronaviruses might just prime ORF-1-specific T cells, since the ORF-1-coded proteins are produced first in coronavirus-infected cells and are necessary for the formation of the viral replicase-transcriptase complex essential for the subsequent transcription of the viral genome leading to various RNA species¹⁸. Therefore, ORF-1-specific T cells could hypothetically abort viral production by lysing SARS-CoV-2-infected cells before the formation of mature virions.:

Reviewer Reports on the First Revision:

(Referee #1)

The authors have satisfactorily addressed my concerns.

(Referee #2)

The revised manuscript is much improved. In particular the added analyses of neutralizing antibody responses provides important additional information about the combined adaptive immune response to SARS-CoV2. I have no additional questions.

(Referee #3)

In this revised manuscript, Le Bert et al do an excellent job responding to all of the comments of the reviewers. This manuscript is an important contribution to our understanding of pre-existing T cell responses to SARS-CoV-2.