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Peer Review Information

Journal: Nature Immunology
Manuscript Title: SARS-CoV-2 antigen exposure history shapes phenotypes and specificity of memory CD8+ T cells
Corresponding author name(s): Joshua Wolf and Paul Thomas

Editorial Notes:

Transferred manuscripts	This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Immunology.
Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A33310-T

Message: 17th Dec 2021

Dear Paul,

Many thanks for transferring your manuscript "SARS-CoV-2 antigen exposure history shapes phenotypes and specificity of memory CD8+ T cells". As discussed we're interested in considering a revision. Please revise the manuscript as outlined in your earlier correspondence and it's probably helpful to re-format the manuscript to NI style at this stage.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file in Microsoft Word format. [REDACTED]. As discussed we'll probably send the manuscript back to 2 of the original Refs. Any q's please ask.

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a

compelling argument. This response will be sent back to the referees along with the revised manuscript.

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We hope to receive your revised manuscript within two weeks. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor

Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

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Author Rebuttal to Initial comments

General response

We thank the Reviewers for the thorough evaluation of our work. While all of the Reviewers found our approach interesting, there were several concerns raised collectively that we would like to address in a general comment.

First, we regret that the conceptual framing of our paper was not sufficiently clear in our original submission. Our aim was to compare the magnitudes, repertoires, and phenotypes of SARS-CoV-2-specific CD8 T cells across distinct antigenic histories, testing the central hypothesis that diverse antigen exposure histories would result in distinct CD8 T cell recruitment as measured by one or more of these parameters (magnitude, repertoire, and phenotype). We have worked diligently to clarify this throughout the revised text and figures. Specifically, in the original submission we compared individuals who had been infected only (inf), vaccinated only (vax2), infected and then received one mRNA vaccination (inf-vax1), or infected and received both mRNA vaccine doses (inf-vax2). We have now more than doubled the number of samples reported (power was also a recurring concern raised by the reviewers), and we have also added a fifth comparator group of great scientific and clinical interest: breakthrough infections, or vaccination followed by infection (vax2-inf). This expanded analysis confirms our hypothesis, as we found that antigen exposure history does indeed lead to divergent CD8 phenotypes. For example, we show that vaccination after infection (inf-vax1 and inf-vax2) drives a clear enrichment of differentiated effector phenotypes when compared to infection or vaccination alone. From our novel analysis of breakthrough infections, we report the surprising result that, despite spike-

specific priming, we observe no magnitude enhancement in spikespecific responses; interestingly, breakthrough case phenotypes also appear most similar to infection-only subjects, effectively as though there were no measurable recall advantage from vaccination in these breakthrough subjects . This is the first report we are aware of that shows such a dramatically divergent phenotype in the T cell compartment associated with breakthrough infections. We believe these expanded data, additional comparator groups, and efforts to clarify the central purpose of the manuscript have greatly improved our work, and we sincerely appreciate the reviewers' careful critiques of our original submission.

In addition to the analysis described above, we have also added a substantial new analysis demonstrating the specificity of our multimer reagents generated by cloning and expressing 12 TCRs in monoclonal cell lines and testing functional and multimer binding responses. Our expanded data set has also resulted in an even larger set of curated epitope-specific TCRs (over 4000 unique clonotypes), which we believe is the largest paired SARS-CoV-2 epitope-specific data set available.

We have made a number of other textual and figure changes and believe we have addressed all of the major critiques of the original review. Thank you again for your efforts, and we look forward to your comments.

Below, we address specific comments raised by each of the Reviewers in a point-by-point response. The original Reviewer's comments are in black; our response is in blue; locations of described changes in the revised manuscript are in red.

Point-by-point response.

Referee #1 (Remarks to the Author):

Major points:

- The authors draw general conclusions by comparing a total of 19 vaccinated individuals (9 of those previously exposed to SARS-CoV-2 infection).
The lower number of subjects as well as the bias in caucasian ethnicity (as shown in Supplemental Table 1) and HLA representations do not allow the application of the current findings to the general population. Inclusion of additional ethnicities in the donor cohorts as well as the inclusion of HLA alleles frequent in different ethnicities behind the one studied would help to extend the authors' findings.

We have now substantially increased the number of subjects in our study (from 19 to 55). Unfortunately, and despite the concerted efforts of the SJTRC study, our cohort does not have sufficient

enrollment of underrepresented ethnicities and rare HLAs to tackle this important problem, which will likely only be addressed by cohorts specifically designed for such questions. Although we fully agree that studying epitope-specific responses in such populations is of great importance (as immunodominant epitopes for those populations remain unknown and thus couldn't be studied with available MHC-multimers yet), we simply were unable to recruit the donors with ethnic backgrounds necessary to address this. However, we have now added a *limitations section* to our discussion, and we have taken efforts to not only emphasize this particular limitation within our study but also to specifically call for broader study of immunodominance in more diverse HLAs both within and outside the context of SARS-COV-2 (Lines 427433).

-The selection of few epitopes (18 total) to represent immunodominance is not shown based on the overall T cell repertoire that a single subject can recognize. This is a limitation of the study and should be clearly discussed in the manuscript

We appreciate this feedback. Although the epitopes we selected were identified as immunodominant in comparison to many other epitopes in source studies, suggesting that at least in some cases they contribute to a large fraction of the total T cell response, we agree that we are unable to exhaustively characterize the response to the entire virus. To address this shortcoming, we have edited the results section to emphasize that we can only compare the magnitude of the response within the selected epitopes (Lines 214-219), and we have also specifically noted this limitation in our discussion (Lines 423-427).

-The expansion of spike-specific clones after vaccination is not observed in 7 out of 9 of the previously exposed donors analyzed (as shown in Fig. S7). The authors instead selected 1 of the 2 only donors with clear spike clonal expansion (Fig 2G). While in Fig 2H they plot an increase of the proportion of spikespecific T cells, it is not clear how it was calculated (ratio of pre-vaccination and ratio post-vaccination?).

We apologize for the lack of clarity in our original manuscript. We have now revised the part of the results describing this point (Lines 233-245) and added an additional within-HLA analysis of epitope hierarchy to make our point more clearly (new Fig. 2f, Lines 226-233). We also reproduced the observed effect on additional donors, increasing the statistical power of the analysis (see Fig. 2h).It is important to note that the simultaneous consideration of both spike and non-spike specific T cell responses helps us to assess T cell boosting while ignoring the possible influence of response dynamics. We expect that between the timepoint after infection and the timepoint after vaccination the proportions of spike and non-spike specific cells relative to each other will remain stable (as memory cells should decline with the same rate independent of their specificity), unless the vaccination triggers a recall response of spike-specific cells. If the vaccination triggers a recall response in the spike specific cells, the proportion of this

response will increase. Depending on the sampling time, this increase can be observed as: 1) the expansion of spikespecific cells with decline of non-spike specific cells (now observed in 9/16 donors); or 2) a stable spikespecific response with decline of non-spike specific cells (Fig. 2g and Fig. S7). The fraction of spikespecific cells was calculated as the ratio of spike-specific cells to all cells in the sample at both prevaccination and post-vaccination timepoints (and this explanation has now been added to the figure legend of Fig. 2h (lines 795-797); and lines 237-238).

Based on those data, it is unclear if additional spike epitopes might indeed be more immunodominant and therefore overcome the initial spike epitopes or if there is not a specific clonal expansion to spike epitopes. To address this point, did the authors assessed the breadth of the repertoire for vaccinees individuals compared with natural infection? Are there any novel spike epitopes induced by vaccination that overcome the previous epitopes recognized in natural infection?

We thank the reviewer for raising this important question. Unfortunately, answering this question fully would require a substantial number of experiments and is well outside the scope of our paper (which is neither designed nor powered to discover or characterize novel epitopes). However, we can begin to address this important question by comparing the diversity of the epitope-specific T cell receptor repertoire across different antigen exposure contexts. As we now show in the revised manuscript (Fig. 3j-k), there is no significant difference in TCR diversity in either spike or non-spike specific epitopes between study groups (Lines 317-320). We have also added a sentence about the possibility of the response to novel epitopes in the vaccine to the limitations section (Lines 425-427).

Minor points:

- Authors interestingly show analysis on the effect of the SARS-CoV-2 variants in their epitopes. It would be useful to specify the sequences they based their analysis on to identify those amino acid mutations.

Viral sequences from the GISAID database (www.gisaid.org/hcov19-variants/) were used for this analysis. We considered only sequences that were present in at least 10% of all sequences in the pango lineage of interest. We clarified this in the methods section (Lines 488-493).

- Harris et al., Journal of Biomedical informatics and Sahin et al., Nature references are duplicated.

We thank the Reviewer for this comment. We checked both references and while they have identical first authors and even journals these are different papers. We have double checked that they are cited in the right spot.

Referee #2 (Remarks to the Author):

Minervina and colleagues have studied convergent CD8 T-cell episode responses, comparing infection to vaccination. The centre of this approach is the application of 18 DNA barcoded HLAI multimers, allowing them to do a range of single cell techniques, including RNAseq TCRseq and CITEseq.
This manuscript is largely focused on the intrinsic interest of the ability to marry the barcoded multimers to the single cell approaches and beyond this, perhaps struggles slightly to assert what is the novel, conceptual point that has been delivered by the process, over and above previously published studies.

We regret the lack of clarity in the original submission. As described in the general response above, our main objective was to explore the effects of distinct antigenic exposure histories (combinations of infection and vaccination) on the magnitude, repertoire, and phenotypes (as assessed by gene expression and surface phenotyping) on the CD8 T cell response to SARS-CoV-2. We have now tried to clarify how our approach provides new information beyond published studies. We hope this case is now more clear with the addition of the breakthrough infection group, which shows a clear divergence in the expected phenotypes and magnitudes of the spike-specific response, which show no evidence of prior priming. In addition, we have added a section in the introduction discussing the relative strengths and weaknesses of various approaches for measuring CD8 responses (Lines 79-85). In particular, the dominant assay used in the large majority of

SARS-CoV-2 studies are functional assays, which only allow a phenotypic characterization after stimulation (and require the cell to perform a specific functional response in a given time window to even be included as a “specific” cell). Our approach, which by necessity does have to focus on a smaller number of epitopes, importantly allows direct *ex vivo* characterization of CD8 T cells and linkage of phenotype, TCR sequence, and magnitude. Linking specificity and high resolution phenotyping, as we do here, would not be directly possible with a peptide restimulation assay.

The title and abstract focus the reader on ‘convergent epitope specific responses’, but this point is something of a straw man, since the insights from this are not especially striking or novel beyond the many epitopemapping studies published through other approaches.

We thank the Reviewer for this feedback, which has helped us tremendously in understanding that the main point of our paper — assessing the specificities, magnitude, and phenotypes of the CD8 T cells following recurrent antigen exposure events — was not clearly articulated in the original manuscript. We have now heavily revised the title, abstract, introduction, and discussion sections in an effort to better contextualize our findings in this conceptual framework.

The Introduction is somewhat jarring in this sense, citing relatively little of the prior literature from the crowded area and talking up the importance of now comparing the complexities of HLA presentation and T cell subsets. Most importantly, the build-up side-steps the central issue of where we should place

CD8 protection – after infection and/or Pfizer vaccination – on the spectrum of correlates of protection. This is rather key since this paper has elected only to consider CD8 responses, while most other keynote papers consider the response more comprehensively across CD4, B cell memory, nAbs etc

We fully agree with the Reviewer that investigation of the immune response to COVID19 is a rapidly evolving field with an enormous number of publications, and we regret that we did not sufficiently contextualize our work in the broader (and important) literature. In an effort to address the issue, we have now incorporated a much broader set of studies into our introduction and discussion, including seminal papers such as Dan et al, *Science*, 2021; Bange et al, Nat. Med., 2021; Grifoni et al, *Cell*, 2020; Reynolds et al, *Science*, 2021, and 22 additional references (ref. 2-9, 11-14, 31, 33-40, 51-54, 61-64, 66, 68). Although we hope that the Reviewer will find these changes satisfactory, we are of course happy to include reference to specific publications that we might have unfortunately missed if the Reviewer has specific suggestions. We've also added a section in the introduction (Lines 63-69) discussing our current understanding of the role of the CD8 T cells in the response to SARS-CoV-2 infection and vaccination. As noted in the answers above, we have also clarified that the purpose of our study is to determine the effects of diverse antigen histories on CD8 T cell biology, rather than a broader investigation of correlates of protection (though we would note that no rigorous correlate of protection — either cellular or serological — has to our knowledge been published to date).

The scope of the study is relatively narrow, comparing 10 positives and 9 negatives,

To address this point we have now included 36 additional donors to the study. This cohort of 55 individuals now includes 16 post-vaccination samples obtained from 16 participants with no previous SARS-COV-2 antigen exposure, 16 pre-vaccination and 44 post-vaccination samples from 30 individuals who had previously recovered from mild SARS-COVID-2 infection, and 9 post-infection samples obtained from 9 individuals who experienced symptomatic breakthrough infections after completing vaccination.

but only including 3 of the possible HLA-A alleles and 2 of HLA-B, these loaded with only 18 of the many described epitopes, 6 of these from spike. Thus, the ability to narrate the quality and quantity of the vaccine response rests on only 6 of the many described epitopes.

In our revised manuscript, we now include a discussion of this issue in the new *limitations* section of the manuscript (Lines 423-429). We have also substantially revised the text describing our epitope selection procedure (Lines 160-169). In particular, we would like to emphasize that the discovery of SARS-CoV-2 epitopes was the focus of multiple large-scale studies that have been published previously (as reviewed by Grifoni et al, *Cell Host and Microbe*, 2021), and we believe that reproducing these studies on new cohorts is outside the scope of our paper, as it is not per se an epitope discovery paper.

Despite these limitations, we think it is important to note that we selected these epitopes because we find that they identify substantial responses in vaccinated and infected individuals. Our average convalescent magnitude across individuals is approximately ~0.4% of the CD8 compartment, which is actually very close to what has been measured with "global" AIM-type assays that use larger antigen arrays (e.g., the entire spike protein). We would respectfully suggest that the gains that come from true epitope-specific characterization, particularly in the ability to query unmanipulated *ex vivo* phenotypes, represent a complementary approach to pooled-peptide stimulation studies.

This gives really narrow coverage of the question. Surely it might have been preferable to opt for a more comprehensive approach, encompassing a larger number of alleles and then starting from TGEM (T cell guided epitope mapping) for an empirical sweep through all epitopes from the entire sequence?Ultimately, this felt like an elegant methodological approach targeted to a specialist CD8 immunology audience that was in search of conceptual questions.

We agree with the Reviewer that our original manuscript regretfully did not sufficiently frame the conceptual advances of our study, and we sincerely appreciate the feedback to this point from the Reviewer. We believe that this feedback, in addition to consultation with the editor, has allowed us to substantially strengthen our revised manuscript. There are several important novel conceptual advances offered by our work, including our findings on the continued recruitment and differentiation of spike-specific CD8 T cells during vaccination after infection, and the surprising lack of spike dominance in breakthrough infections, with little phenotypic evidence of a recall response. In contrast, we show that a novel non-spike-specific CD8 T cell memory is formed after the breakthrough infection.

In addition, we have revised the manuscript in hopes of emphasizing that it is not an epitope discovery study, but rather an in-depth characterization of CD8 T cell repertoire recruitment, expansion, and differentiation after diverse antigen exposures. The method proposed by the Reviewer requires precise loading of hundreds to thousands of diverse peptides in multiple MHC molecules. While it has been used in many demonstration studies, these peptide exchange approaches raise many technical issues and, to do at the scale of our cohort, would require several hundred thousand dollars for reagents alone. These reagents would then require extensive validation due to the issues astutely raised by Reviewer 3. However, we have been able to extensively validate the results of our approach informatically as well as by cloning and expressing 12 TCRs and demonstrating their specificity against our multimer reagents (Fig. S17, S18); to our knowledge, this is one of the largest such validations using this approach.

Among these was the suggestion that vaccination draws on the same specificities as natural infection – predicted from the papers cited here on vaccine enhancement by prior infection. There is description of

the 'most immunodominant epitopes' (line 184) but this is so loosely defined it's hard to catch the implied meaning or formal proof.

Upon reflection, the Reviewer's critique of our use of the term "immunodominance" is spot-on, and we appreciate the opportunity to address this. We have taken efforts to be more specific throughout the manuscript and remove claims about the relative immunodominance of any epitope to the entire response (Lines 216-219).

Since 4 of the epitopes have been selected on the basis of homologies to HCoV sequence, there is potential to weigh into the crowded and now contentious area of prior HCoV cross-reactivity as protective, neutral or deleterious in COVID-19 protection. Again though, beyond using the technology to note the genuinely cross-reactive clonotypes, the approach and samples are never applied to the accrual of any new conceptual advance that take the reader beyond the points being made some months ago by Mateus at al in their Science paper, that cross-reactive clones exist.

We agree that there were papers pointing out the existence of CD4 cross reactive cells (Mateus et al, *Science* 2020; Braun et al, *Nature* 2020; Grifoni et al, *Cell* 2020); however, whether CD8 cross-reactive cells exist and contribute to the response *in vivo* has been less certain. We also want to emphasize that though it is not the main point of the paper, the formal proof of TCR crossreactivity (isolating a specific TCR and proving its specificity to both common cold coronavirus and SARS-CoV-2 epitope variants) was missing from these papers. For instance, Mateus et al, though citing 'epitopes' in the title, only uses peptide restimulation assays, which by definition cannot be used to define an epitope or epitope-specificity.

Referee #3 (Remarks to the Author):

The Introduction claims that "the magnitude of T cell responses in naive individuals following infection or vaccination as well as the effect of vaccination on pre-existing memory cells remains controversial", although I'm not certain what the controversy is.

We sincerely regret the lack of clarity in our original framing of the core question of the manuscript, which is to explore how antigen exposure history and context can influence the phenotypes and specificities of CD8 memory T cells. As noted in the general response, we have substantially revised the introduction section to more clearly define this question (Lines 63-74).

The cohorts here are far too limited and opportunistically constructed to definitively resolve it. There are only 9 subjects in the naïve cohort and a combined 10 individuals in the two "recovered" cohorts.

To address this point, we have now included 36 additional donors to the study. This revised cohort of 55 individuals now includes 16 post-vaccination samples obtained from 16 participants with no previous SARS-COV-2 antigen exposure ("naive donors"), 16 pre-vaccination and 44 post-vaccination samples from 30 individuals who had previously recovered from mild SARS-COVID-2 infection, and 9 post-infection samples obtained from 9 individuals who experienced symptomatic breakthrough infections after completing vaccination. Importantly, we were able to reproduce all the major conclusions from our original manuscript with the addition of this revised cohort, in addition to the novel findings related to breakthrough infections that are now included.

Furthermore, the range of the time between the 2nd vaccination and the main (and sometimes only) sample collection ranges between ~25-60 days, and across this range, it's far from certain that steady-state memory has been achieved, raising concerns about comparisons in this limited cohort.

We agree that the range in sampling is one of the limitations of our cohort, and have added mention of this to the limitation section (Lines 437-441). Importantly, to avoid erroneous conclusions, we rely on comparisons of the *proportions* of the spike (elicited by both infection and vaccination) vs. non-spike (elicited by infection only) T cell response rather than total frequencies, allowing us to minimize the possible influence of response dynamics (Lines 237-242).

Furthermore, all of the subjects in the recovered cohorts received 2 vaccine doses, and in only 3 of them were samples measured between the 2 doses. Finally, the emerging clinical standard seems to be that convalescent subjects should get only 1 vaccine dose (probably at least 3 months post clearance of the infection), so the relevance of this data is somewhat reduced.

We want to thank the Reviewer for this valuable comment. To address this issue, we added 7 additional SARS-CoV-2 donors who were sampled after the first and after the second doses of the vaccine, substantially increasing our sample size for this question specifically (N=10). Indeed, we observed that most of the changes in epitope-specific T cell distribution between phenotypes and specificities happen already after the first dose, though it seems that a subset of individuals may still show increases in both T cells and antibodies after the second dose. See Lines 151-155, 245-247, Fig. 2f, S1, S8.

The primary concern is about the quality of the dextramer reagents, and this stems from the bivariate flow plot in Figure 1A (this panel really should be broken up into 2-3 separate panels) . Simply put, the intensity of the dextramer stain and the separation of it from the bulk population really aren't very good. Only one such plot is provided (it appears to be recapitulated in SF3), and a supplementary figure should be prepared that shows the bivariate flow plots for all subjects and all sorts.

During the original manuscript preparation, we unfortunately only obtained flow files with limited total acquisition counts. However, as suggested by the Reviewer, we revised this approach for all samples added during the revision. We now show these total event sort files in Fig. S2 with representative flow plots for individuals with the same HLAs (and hence the same dextramer pool) but who show different frequencies (low and high) of the dextramer staining. We believe that this panel shows the full spectrum of the data without occupying too much space (as we now have more than 80 samples). Importantly, most of the reported results are independent of the flow results, as our analytical approach utilizes proportions of epitope-specific responses rather than flow cell counts (as they can be arbitrary and depend on personal preference of gate placement).

There is an important related secondary issue. The results in the flow cytometry assays combine all the various multimers in a single channel, and while there is a good reason to do this, it also means that without additional experiments, it is impossible to evaluate the quality of each member of the pool.

Although identifying good controls for MHC-multimer reagents has been a perennial problem for the field, we have validated our findings using reverse genetics, and we now include those experiments in our revised manuscript. For each of the epitopes resulting in a large TCR similarity cluster, we generated a T cell line expressing one or two of the central TCRs from a public sequence motif (Supplementary Table 7). We then used these T cell lines with monoclonal specificity to benchmark our MHC-multimers (Fig. S18). The frequency of dextramer positive populations varied between different cell lines, but we believe that this difference was mostly due to the Jurkat cell line variable TCR expression rather than the quality of the dextramer reagent, as the exact same A01_dextramer_TTD stained TCRline1 and TCRline2 with different intensity (third row Fig. S18). In total, we generated 12 T cell lines, and all TCRs associated with a dextramer in primary cells bound that particular dextramer in the Jurkat analysis. We also used peptide stimulation to independently confirm the specificities of generated T cell lines (Fig. S17). The performed experiments have confirmed that our dextramer reagents can successfully stain Jurkat cell lines with a cognate specificity with low background noise, importantly demonstrating both sensitivity and specificity.

Next, while high-throughput methods such as these can tolerate false-negatives due to the concerns I've raised here, the limitations of the approach need to be explicitly stated, and claims of immunodominance based upon use of these reagents such as in lines 184-188 should be considerably more tempered. Finally, the frequency of most of the responses measured is really low, and probably near the noise limit of the technology. Out of perhaps 100 measurements in Figure 2e, only about 12-13 are above 0.1% of CD8+ T cells, and many are below 0.01%.

We appreciate this feedback and agree that we must reconsider our claims of relative immunodominance across HLA types. We have revised our manuscript accordingly, taking efforts to be

more specific throughout the manuscript and removing claims about the relative immunodominance of any epitope to the entire response (Lines 216-219). However, we do compare relative responses to specificities within an HLA type, for example clearly showing the shift within A01 and A02 carriers towards spike-specific responses after vaccination (Fig. 2f, S6, Lines 226-233).

To ensure the specificity of our staining, we set a threshold of at least 4 UMIs per best dextramer to be considered positive, rather than considering all sorted cells to be epitope-specific; because we entirely agree that there are likely some false-positives in the sorted population, the "total sorted population" value is not used in the majority of calculations. However, we want to note that, even in donors with a low frequency of response/low number of sorted cells, we observe TCRs from the biggest TCR sequence similarity motifs (e.g. B8, R16, R3, R14, N1, R30, Fig 4c), again indicating that multimer staining assay is quite specific.

To me, the most intriguing data in the paper comes from sequential measurements of responses at 2 timepoints after the second dose, as seen in Figure 3h-j. This data suggests that shortly after vaccination of recovered subjects that an antigen-specific population of T cells emerges with a T(EM-Ex) phenotype that decreases in frequency over time. Other data suggests that this population contains expanded clonotypes (lines 239-241 and SF10). Unfortunately, the legend to Figure 2h does not tell us how many subjects have T cells with that phenotype, and tracing back from here to Figure 1a, it's possible that nearly the entire population coes from a single sample from a single donor (R9, though I've had a hard time squaring that with Figure 4 and Supplementary Table 2, though it does seem confirmed by SF8). If this cluster is indeed nearly all from one subject at a single timepoint, it further highlights the limitations of the cohort that I noted in the second paragraph.

We have now expanded the cohort to include more individuals with the relevant timepoints and can report that the phenotype is robust across multiple individuals (Fig S13a, b). Indeed, a lot of cells from this cluster originate from a single donor (R9, which is now renamed to R29 to preserve consistency within groups). However, on Fig S13b we show that the observed decline is reproducible within multiple donors. Moreover, on Fig3i where each dot corresponds to a single sample, the correlation is significant after the exclusion of any particular donor.

While UMAP plots such as in Figure 3a combining all data from all subjects can be visually stunning, they can also be misleading unless additional plots are provided that deconvolve the data on a per subject and per timepoint basis as in Figure 3h (but done with more clarity).

We have now provided UMAP plots split by cohort and specificity (Fig. S10), timepoint (Fig. S13a), and epitope (Fig. S11). As visualizing with per-donor/timepoint UMAPs would require 80 plots, we have instead included a per-donor bar plot that we believe conveys the relevant information (Fig. S9).

Importantly, while there is of course a substantial variation in both the relative distribution of cells across clusters and the number of cells acquired from each donor, all donors contribute to multiple clusters (and vice versa). We also provide all raw data (including UMAP coordinates for each cell and correspondence of each cell to a donor/timepoint) in Supplementary Table 5.

- Results
- The reporting of the timepoints on line 114 is both incomplete and misleading. The numbers are give as ± x, without telling us what x is. Furthermore, the ranges in S1 are actually quite large.

The manuscript has been revised throughout to include not only mean value ± SEM, but also the range (Lines 178, 461-464). We also included a sentence about sampling timepoint range in the limitation section (Lines 437-441).

- Line 142 refers to Supplementary Table 2, but it should be Supplementary Table 1.

We thank the Reviewer for this comment. We initially referenced table 2 as it contained the HLA information for each donor, we've included links to both tables for this sentence now. See Lines 171-173

- Line 163 states that "Eleven of the most abundant clonotypes matched a single specificity across all cells (Fig. 2B)," but no hint is given of the number that don't match (one). Please revise.

This part has been revised to address this issue. Please see Lines 192-203.

- Comments on immunodominance should be tempered, making note that the quality of each of the reagents in each pool has not been validated.

We revised the manuscript throughout to limit claims regarding immunodominance. We also have added the validation of substantial part of our reagents using generated T cell lines.

- A reference is needed for Francis et al on lines 296-298.

This reference has been added.

- Figure 1
- Figure 1 - Panel A - there is a discrepancy in # of naïve then vaccinated donors in the figure itself and in the legend (N=10, but 9 green triangles).

The number of the donors indicated on Fig1a in each cohort has been revised.

- Figure 1 - specify units for RBD IgG in panel B. It is also not described in methods section either (Lines 537-566), though it should be. Same for Figure S2, S6,

Lines 694-703 were added to the methods section. The description was also added to the figure legends of Fig. 1c, Fig. S1, Fig. S5 and the indication of the units used (Normalized OD) was added to the figures.

- Figure 2
- Panel A - put units on UMI counts (it is there in S4).

The UMI units were added to Fig2a.

- Panel B - clone in the second row bound more than one unrelated multimer. Please comment.

While we put a lot of effort in cleaning the 10x Genomics artifacts, the technology is not perfect and generates some noise. For example, it is likely that some cells within the mixed clonotype are a doublet rather than a single cell, and the UMI counts for dextramers come from the second cell in a droplet. But as we then use the nucleotide sequence of the strong majority TCR clonotype to expand the epitope assignment, this technical limitation should have little to no effect on further analysis and will not include false positives in our clonotype counts. The fact that TCR similarity clusters on Fig4 have largely the same assigned specificity additionally confirms that there are no significant issues with the use of dextramer staining with subsequent barcode sequencing to define TCR specificity.

- Panel D - instead of using the dextramer platform, this panel uses barcoded tetramers prepared using Biolegend reagents as described in lines 525-535. Although it doesn't really matter because the data in the panel are convincing, there was no need to use the bar-coded reagents because the readout was conventional flow cytometry, and the description of the assembly of these reagents is useless because the concentrations of the components are not included.

We thank the Reviewer for pointing this out. The methods section is now revised to include all relevant concentrations (Lines 662-666). We agree that for this particular experiment there was no need to use barcoded reagents; this decision was made based on the immediate availability of these reagents.

- Panel E - suggestion - instead of using color to code for HLA allele, (which is noted on x-axis anyway), use color to indicate cohort.

We thank the Reviewer for the suggestion. In this figure panel we wanted to emphasise that comparison of the magnitude of response should be made within an HLA (as the same reagents were used for staining of each individual), rather than comparing across all HLA types. However, we also added different dot shapes on Fig. 2e to indicate the cohort.

- Panel G - the wedges have some weird artifacts that were initially distracting. Same with S7.

We thank the Reviewer for pointing this out. To avoid these artifacts, we have changed the type of visualisation used for Fig. 2g and Fig. S7.

- Panel H - the claimed result is probably true, but there's a wide range of timing intervals, probably too much to make a conclusion based upon only 6 donors.

We added 9 new SARS-CoV-2 recovered individuals to our cohort (N=16). With the addition of the new samples we were able to not only reproduce our original result, but also increase the significance.

- Figure 3 - It is really hard to interpret this data without somewhere more clearly breaking out the cells by cohorts and even individuals. This also applies to the timepoints in panel H, which must be specified but are not.

We added supplementary figures S10, S11 that show individual UMAP plots for different sample cohorts and a bar plot for individual cluster distributions (Fig. S9). Timepoints on the panel: correspond to circles and triangles on Fig. 1b (Timeline). Depending on the sample group they might be taken pre or postvaccination, but in all cases have at least a month in between. We clarified it in the figure legend S13; see Lines 897-898.

- Figure S3.
- Why was staining panel so minimal? Furthermore, the methods do not appear to contain any information about the instrument used for sorting.

To address this issue we included representative staining panels for individuals with the same HLA (same dextramer mix), but varying frequencies of response (Fig. S2). We also included staining results for all generated T cell lines in a dextramer staining and peptide stimulation experiment (Fig. S17, S18). The methods section was revised to contain the information about the instruments used for sorting and flow experiments (Lines 516, 626, 657-658).

- Figure S5
- There are two very weird things about the cell lines in this figure. First, it's impossible to tell iff the cells displayed are mCherry+ or mCherry- (perhaps internal untransduced cells should have been included). Second, the responder frequency is very low, suggesting something is amiss.

Indeed, the intensity of mCherry on these flow plots is very low. In this experiment, anti-TCR antibody conjugated with PE was also a part of this staining panel. To increase the separation between these two overlapping fluorophores (mCherry and PE), we had to set mCherry voltage quite low, resulting in a low intensity on these plots. To address this issue, we used this T cell line on Fig. S17 (the peptide stimulation experiment that didn't have PE in the staining panel), where it is obvious that mCherry expression in these Jurkats is actually very high.

The IFNgamma cytokine expression is indeed quite low by this Jurkat cell line in comparison to what might be expected for primary cells. This is a known issue with this cell line, as even the PMA stimulates IFNgamma production in only 5% of cells. However, the endogenous NFAT-GFP reporter results in a high responder frequency with very low background in both peptide stimulation experiments (Fig. S4, S17)

- Fig. S8. the color code for clusters should be recapitulated here — it would make it easier for the reader.

We thank the Reviewer for this comment. Color codes are now reproduced in all stand alone figures (Fig. 3d, S9, S10, S11, S13).

Decision Letter, first revision:

Subject: Your manuscript, NI-A33310A

Message: Our ref: NI-A33310A

9th Feb 2022

Dear Dr. Thomas,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "SARS-CoV-2 antigen exposure history shapes phenotypes and specificity of memory CD8+ T cells" (NI-A33310A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments and please make sure to upload your checklist.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "SARS-CoV-2 antigen exposure history shapes phenotypes and specificity of memory CD8+ T cells". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Immunology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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If you have any further questions, please feel free to contact me.

Best regards,

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Reviewer #1:
Remarks to the Author:
I want to thank the authors for addressing in a satisfactory way what was by far the largest concern with the previous version of the manuscript — the issue of anamnestic responses to breakthrough infections in vaccinees. I hope that the authors will continue to explore this issue in depth, as it's interesting and important, but it does require more data than currently in hand.

Any remaining concerns I have about the manuscript are minor and the need to address them does not out-weigh the need for timely communication in this field.

Author Rebuttal, first revision:

Reviewer #1:
Remarks to the Author:
I want to thank the authors for addressing in a satisfactory way what was by far the largest concern with the previous version of the manuscript — the issue of anamnestic responses to breakthrough infections in vaccinees. I hope that the authors will continue to explore this issue in depth, as it's interesting and important, but it does require more data than currently in hand.

Any remaining concerns I have about the manuscript are minor and the need to address them does not out-weigh the need for timely communication in this field.

We want to thank the Reviewer for a positive evaluation of our revised manuscript.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A33310B
Message: In reply please quote: NI-A33310B

Dear Dr. Thomas,

I am delighted to accept your manuscript entitled "SARS-CoV-2 antigen exposure history shapes phenotypes and specificity of memory CD8+ T cells" for publication in an upcoming issue of Nature Immunology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Immunology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required. Note - we will aim to fast-track your paper as its about COVID-19.

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