nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: Differentiation of exhausted CD8 T cells after termination of chronic antigen stimulation stops short of achieving functional T cell memory
Corresponding author name(s): Georg M. Lauer, Pierre Tonnerre

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A29982

Message: 11th Aug 2020

Dear Professor Lauer,
Apologies for the delay getting back to - these are unfortunately very trying times to get papers reviewed.

Your Article, "Differentiation of exhausted CD8 T cells after termination of chronic antigen stimulation stops short of achieving functional T cell memory" has now been seen by 2 referees. You will see from their comments copied below that while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the manuscript for publication, but would be very interested in considering a revised version that addresses these concerns.

If you choose to revise your manuscript taking into account all reviewer and editor comments, please highlight all changes in the manuscript text file.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

If 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.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/ni/authors/index.html. Refer also to any guidelines provided in

this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

The Reporting Summary can be found here:
https://www.nature.com/documents/nr-reporting-summary.pdf

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

You may use the link below to submit your revised manuscript and related files:
[REDACTED]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. 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.

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.

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

Thank you for the opportunity to review your work.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

The Macmillan Building
4 Crinan Street
Tel: 212-726-9207
Fax: 212-696-9752
z.fehervari@nature.com

Referee expertise:

Referee #1: T cell dynamics, exhaustion

Referee #2: HCV, host response

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
GENERAL COMMENTS

Our knowledge on the effect of antigen removal on T cell exhaustion has thus far largely been limited to murine models. However, with the advent of Direct Acting Antivirals (DAA), a chronic human viral infection – HCV- can now be cured, providing an ideal set-up to study this phenomenon in humans.
Having access to a cohort of chronically HCV infected individuals pre-and post DAA, the authors compared the phenotypic, functional and transcriptomic features of tetramer+ HCV-specific CD8+ T cells before and after HCV cure. They also investigated spontaneous HCV resolvers, allowing for comparison of therapeutic vs immunological cure of HCV. As expected, the HCV-specific cells during chronic infection expressed typical features of exhaustion, such as high expression of inhibitory receptors, high EOMES and low TCF-1 and T-bet, and low cytokine production (but high cytolytic capacity) in comparison to cells from resolvers. Antigen removal by DAA led to decrease of several exhaustion-associated features in HCV-specific CD8+ T cells of previously chronically infected participants. Compared to HCV-specific CD8+ T cells from resolvers, while DAA-mediated viral cure led to phenotypic and transcriptional changes towards a memory-like profile, the authors identified "immunological scars" that are not reversed, such a low functionality and persistent alterations in expression of specific transcription factors.
Overall, it is an important and well-designed study, with important results that may have broad implications for chronic human diseases, in particular infections and cancers.
Addressing the weaknesses mentioned below would further improve the manuscript. The strong statements made on the newly identified transcription factors that remain differentially expressed after DAA should be backed up by some validation experiments in some additional subjects. There is otherwise a risk for spurious associations.

SPECIFIC MAJOR COMMENTS

1. FIG 1: The explanation of the cohort is nice and easy to grasp. Can the authors summarize the frequency of HCV-specific CD8+ T cells they detect in their different cohorts (for example, chronic pre DAA vs resolvers)? How well are these HCV-specific CD8+ T cells preserved over time for the treated cohort and the resolver cohort?
2. Fig 1, Ext data Fig 2: The definition of "partial escape" is quite vague. What criteria were used? Also, the interpretation of the data is more difficult here. It is not certain that these responses are actual escape, could they correspond to sequence variants in the infecting strain? Overall, the results on this category of epitopes are less contributive to the manuscript than the strong data obtained on T-EX and T-F-ESC.
3. FIG 3f : What are the main variants which contribute to PC2, and thus explain the notable separation there is between the Tmem and all other HCV-specific CD8+ T cells? If the Flu-specific CD8+ T cells (acute, quickly resolved viral infection) from the treated HCV group was added to the PCA, would they cluster with the Tmem or the other HCV groups? Similarly, what variables contribute to PC1 and relate to the "correction" of the exhausted HCV-specific CD8+ T cells with DAA?
4. FIG 5 : Can the authors also add the timeline for CD107a, to see how a functional feature associated to exhaustion is preserved over time. Exhausted cells do not gain cytokine production – do they at least maintain their cytotoxic capacity?
5. FIG 5: Should data on the duration of chronic infection be available, it would be interesting to see whether the longer a person has been infected with HCV, the more "stubborn" the defects are. This reviewer realizes that it would only be "documented" HCV infection and that this parameter can be hard to reliably define, however.
6. FIG 6 : Can the authors discuss the difference in clustering between the PCA of figure 3f and that of Fig 6? In Fig 3f, the authors noted that the memory differentiation status, the activation profile and even some transcription factors (TF) normalized; what other pathways then explain the greater clustering of Tex post DAA with cells from resolvers than with Tex pre-DAA?
7. FIG 6 : There are 224 differentially expressed genes (DEG) in the Tesc pre vs post DAA, 176 of which were common with the Tex group. Can the authors provide details on these DEGs and the pathways they are implicated in?
8. FIG 6 : The authors identify 6 additional TF which, like TOX, maintain a differential expression in Tex after DAA in comparison to Tmem. While the FDRs are low in the GSEA analyses presented, these results are robust because they leverage co-expression of a number of genes; however, the authors make strong statements based on the differential expression of individual genes. There is a risk for spurious associations here given the limited size of the cohorts. It would be important to confirm these results in a small "validation" cohort.
9. Does the expression of these TF correlate with that of TOX, suggesting a common mechanism of regulation?
10. Throughout the paper, the CD8+ T cells specific for an HCV epitope which has escaped (TF-ESC) were a nice intra-donor nice comparative of an exhausted vs non-exhausted HCV-specific CD8+ T cells. However, when the epitope changed to escape, a new set of HCV-specific CD8+ T cells are primed in a highly inflammatory milieu, resulting in some phenotypic and functional differences (Snell et al., Immunity 2018). Could this phenomenon also participate in the difference observed between Tex and Tesc?

MINOR COMMENTS

1. Fig 6e : Is the FDR in red an absolute 0?
2. In the discussion, the link between the study and checkpoint inhibitor blockade is a little unclear; after all, ICB do not work by removing antigen. Can the authors highlight the importance of their study from other angles? Their results are more broadly relevant indeed.
3. Line 295 – 298 could be misleading; it is an accurate statement if the authors are referring to the phenotyping changes between Tex Pre vs Post-DAA only. However, if we take into consideration the transcriptomic data, because there is a number of DEG between Tesc Pre vs Post-DAA, it is hard to associate the differences "overwhelmingly" to TCR stimulation rather than the chronic inflammatory milieu. The wording could be nuanced
4. Typo in line 314 : " […] Tesc [instead of Tex] can still recover by antigen removal alone, in contrast to Tex […]"

Reviewer #2:
Remarks to the Author:
Tonnerre et al. "Differentiation of exhausted CD8 T cells after termination of chronic antigen stimulation stops short of achieving functional T cell memory"

This study uses complex flow cytometry combined with transcriptional analysis and NGS of viral sequences to assess to which degree exhausted hepatitis C virus-specific CD8 T cell populations recover from exhaustion after treatment-induced eradication of the virus. This question is timely and clinically relevant (for the development of immunotherapy for cancer and other chronic infections). HCV infection is a perfect model because it can now be completely cleared by a short (12-24 weeks) course of antiviral treatment. The authors have a unique set of samples (lymphaphereses from 20 patients at multiple time points before and up to 3 years after treatment-induced HCV clearance) and compare the results to a matching follow up samples after spontaneous HCV clearance. The analysis is rigorous and the results are beautifully presented and (for the most part, see specific comments) clearly described.

The study has two main findings:
First, studying phenotypic markers the authors find that 23/37 molecules are expressed at significantly different levels before and after treatment induced HCV clearance. They find a complete reduction in T cell activation (complete loss of CD38, HLA-DR, ICOS, CD69 and CD71 expression), a switch toward a central memory phenotype with more cells expressing CCR7 and, especially, CD127, and a switch towards a higher frequency of TCF-1 than Eomes-expressing cells. However, function is not recovered, and critical transcriptional regulators remain mostly fixed in the exhaustion state. The results of HCV-specific CD8 T cells are compared to those of influenza, CMV and EBV-specific CD8 T cells in the same samples, and to HCV-specific CD8 T cells from patients after acute HCV infection. A subset of the patients is followed at additional time points up to 3 years after treatment-induced HCV clearance to assess long-term effects.
This part of the study is consistent with previous reports (Wieland, Nat Commun 8, 15050 (2017); Alfei, Nature 571, 265-269 (2019)) but of much greater scope than these previous studies.

Second, the authors compare HCV-specific T cells that target epitopes without viral escape, with partial viral escape and with full viral escape. They report that the phenotype of T cells that target viral escape mutations was functionally and transcriptionally similar to that of memory T cells from spontaneously resolved acute HCV infection. Because viral escape mutations typically occur early in infection, the authors conclude that these T cells have been exposed to their cognate antigen for a shorter duration than those that target conserved epitopes. This would indicate that there is a window of opportunity early in chronic infection therapies to rescue from T cells from exhaustion.

While I find this second hypothesis intriguing, I have multiple questions:

Extended data figure 2 describes how T cell responses are classified into 'exhausted', or targeting epitopes that have 'partially escaped' or 'fully escaped'. The classification is based on viral diversity in each patient as assessed by next generation sequencing. If I understand the approach correctly, T cell responses are first mapped with a panel of common HCV genotype 1a epitopes (multimer staining) and the sequence of these peptides is then compared to corresponding HCV sequence of the patients. Any difference is defined as mutation (indicated by red letters in panel A). If 100% of the viral sequences from the patient match the sequence of the common HCV genotype 1a epitope, this specific T cell response is classified as 'exhausted'. If 100% of the viral sequences from the patient differ from the sequence of the common HCV genotype 1a epitope, this specific T cell response is classified as 'fully escaped'. If there is sequence heterogeneity in the patient for the epitope of interest, this specific T cell response is defined as 'partially escaped'. In a second part (panel B), T cells stimulated with the respective peptides and IFN-g production after stimulation with variant peptide is compared to IFN-g production after stimulation with common HCV genotype 1a epitope. The relative decrease in IFN-g production is reported.

1. Legend to Extended data figure 2: Please add missing word (x): 'Recognition of variant peptide compared to (x)…..'

2. I find it difficult to extrapolate from viral sequence to T cell recognition: Case 115 =(KLVALGINAV) is defined as fully escaped, but there is almost no decrease in IFNg production when variant and wildtype are compared in panel B.

3. It is impossible to know whether patients with partially escaped or fully escaped epitopes actually encountered the wildtype epitope in the acute phase. Alternatively, it is possible that they were infected with a different strain of HCV genotype 1a from the beginning. Without such data, the conclusion 'The idea that duration of antigen stimulation, rather than duration of recovery, is the defining factor for the ability to differentiate into TMEM' should be modified.

4. NGS data show a mix of HCV sequences for many patients. Do the authors propose that 'partial escape' occurs because the cognate antigen is 'diluted' due to the presence of variant sequences? It is still possible that both sequences are presented to T cells on the same antigen-presenting cell – wouldn't the T cell then receive the full stimulation?

5. The authors analyze sequence diversity at the antigen level (HCV) but not at the TCR level. Different TCRs have different avidity to the respective antigen and it should be

considered that change in phenotype and transcriptome after viral clearance is due to specific expansion of cells with specific TCRs within the epitope-specific T cell population. Unfortunately, both flow cytometry analysis and RNAseq analysis are limited to the 'bulk' population of peptide-specific T cells. I think it is important to add CiteSeq combined with TCR analysis. This would add novel information and can be done immediately as the authors have cryopreserved PBMC from the lymphaphereses.

6. The radar plots show 14 paired samples (pre- versus post HCV clearance) for exhausted CD8 T cells and 8 paired samples for partially exhausted CD8 T cells. Are these from the same patients?

7. Figure 3E: T-SNE analysis is based on the expression levels of CD38, HLA-DR, PD-1, CD39, TIGIT, CCR7, CD45RA, Integrin-Beta-7 and CD62L, but PCA (Figure 3F) is based on expression level of 37 molecules. How do the data from panel E look in a PCA?

Other comments:
8. Figure 1 describes a large panel of EBV, Flu and CMV epitopes, but data are currently only shown for a single patient in Fig. 1D. Please include data on the phenotype of EBV, Flu and CMV-specific T cells in Figure 1C to allow direct comparison with the phenotype of HCV-specific T cells.

9. The methods section contains a sentence that or previously generated T cell lines were used for some experiments. Please explain where in vitro expanded T cell lines (rather than ex vivo studied CD8 T cells) were used.

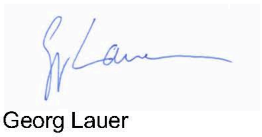
Author Rebuttal to Initial comments

Dear Editors and Reviewers:

We greatly appreciate the positive reception of our manuscript and especially the many constructive suggestions and questions. While the pandemic initially slowed down our efforts, we were able to perform extensive additional experiments and analyses as requested and are delighted that the additional work increased the amount of novel and valuable information and further strengthened our conclusions. We are optimistic that you will agree with this assessment and find this work now acceptable for publication in Nature Immunology.

Please find our specific responses point-to-point below.

With best regards



Georg Lauer

Reviewer #1:
SPECIFIC MAJOR COMMENTS

1. FIG 1: The explanation of the cohort is nice and easy to grasp. Can the authors summarize the frequency of HCV-specific CD8+ T cells they detect in their different cohorts (for example, chronic pre DAA vs resolvers)? How well are these HCV-specific CD8+ T cells preserved over time for the treated cohort and the resolver cohort?

We have added the frequency information to the manuscript (**Extended Data Fig.1d**).

Regarding the resolver cohort, we chose a relatively early time post infection in order to compare T cells with a similar interval between last TCR signal and phenotyping. At this point it is not unusual for populations to further decline in frequency, though the most dramatic contraction happens typically early after virus is controlled.

2. Fig 1, Ext data Fig 2: The definition of "partial escape" is quite vague. What criteria were used? Also, the interpretation of the data is more difficult here. It is not certain that these responses are actual escape, could they correspond to sequence variants in the infecting strain? Overall, the results on this category of epitopes are less contributive to the manuscript than the strong data obtained on T-EX and T-F-ESC.

We realize that this definition was not explained in the best way. The definition of partial escape was the IFN-g response to the variant being diminished to less than 75%, but more than 25% compared to the response elicited by the standard epitope sequence (full escape being defined as zero or maximally up to 10% of the response size with wild-type peptide). We completely agree that "partial escape" is less straightforward to interpret than "full escape" and therefore have limited our comparisons to preserved versus full escape for almost all analyses. Having said that, we thought it was relevant to show the data in **Fig.1c** as it shows these cells with an intermediate

or mixed phenotype between the two more clearly defined groups of responses (fully preserved versus fully escaped), supporting that differences in TCR stimulation are key to the observed phenotypes. We also have discussed the classification in additional detail in a response to Reviewer #2.

3. FIG 3f : What are the main variants which contribute to PC2, and thus explain the notable separation there is between the Tmem and all other HCV-specific CD8+ T cells? If the Flu-specific CD8+ T cells (acute, quickly resolved viral infection) from the treated HCV group was added to the PCA, would they cluster with the Tmem or the other HCV groups? Similarly, what variables contribute to PC1 and relate to the "correction" of the exhausted HCV-specific CD8+ T cells with DAA?

These were very helpful suggestions and we have now added the flu responses to the PCA analysis and included the variables driving the differences in each principal component (**Fig.3f** and **Extended Data Fig.3e**). Interestingly, while the overall message from the figure remains unchanged, the addition of influenza responses slightly changed the principal components (based on influenza cells being memory with a slightly different phenotype compared to naturally resolved HCV) and now therapy-related changes are reflected in both PC1 and PC2.

4. FIG 5 : Can the authors also add the timeline for CD107a, to see how a functional feature associated to exhaustion is preserved over time. Exhausted cells do not gain cytokine production – do they at least maintain their cytotoxic capacity?

We have added longitudinal data for CD107a and CD69 in **Fig. 5** and in a new Extended Data Figure (**ED Fig. 4b**). Overall, many more cells are activated (CD69+) and mobilize CD107A compared to the number of cells secreting cytokines and this general pattern remains throughout. We also added in the main **Fig. 4** an analysis of the polyfunctionality of these cells. This shows that most polyfunctional cells with cytokine co-production functions are absent in T_{EX} as compared to T_{ESC} or T_{MEM}. However, CD107a+ cells remain detectable in T_{EX}, suggesting that cytotoxicity is less impacted in these cells.

5. FIG 5: Should data on the duration of chronic infection be available, it would be interesting to see whether the longer a person has been infected with HCV, the more "stubborn" the defects are. This reviewer realizes that it would only be "documented" HCV infection and that this parameter can be hard to reliably define, however.

This is indeed a great question, and as the reviewer states, it is unfortunately very difficult to ascertain. Overall, we can firmly say that all but one patient were infected for at least a decade, and even the one exception was close to 19 years post documented exposure. We are trying to study patients getting treated during acute HCV infection, but this is challenging for the obvious reasons.

6. FIG 6 : Can the authors discuss the difference in clustering between the PCA of figure 3f and that of Fig 6? In Fig 3f, the authors noted that the memory differentiation status, the activation profile and even some transcription factors (TF) normalized; what other pathways then explain the greater clustering of Tex post DAA with cells from resolvers than with Tex pre-DAA?

We would like to suggest that it is not too surprising that an analysis based on protein expression of only 37 pre-selected variables mostly relevant to T cell exhaustion (**Fig. 3f)** yields a slightly different result compared to an unbiased approach testing gene expression across the whole

genome. The fact that both T_{EX} post DAA and T_{MEM} share the absence of TCR stimulation would be expected to lead to an overall more similar profile in the much broader RNAseq profiling, as seen in **Fig. 6**.

7. FIG 6 : There are 224 differentially expressed genes (DEG) in the Tesc pre vs post DAA, 176 of which were common with the Tex group. Can the authors provide details on these DEGs and the pathways they are implicated in?

We added this important information in a heatmap as **Extended Data Fig. 5c**. Overall, while these genes were indeed differentially expressed in both T_{EX} and T_{ESC}, the figure shows that differences were rather negligible in the T cells targeting escaped epitopes. As it seems questionable whether these small differences are truly reflective of a major biological change of the T_{ESC} we would prefer to just present the information in the new figure without drawing strong conclusions.

8. FIG 6: The authors identify 6 additional TF which, like TOX, maintain a differential expression in Tex after DAA in comparison to Tmem. While the FDRs are low in the GSEA analyses presented, these results are robust because they leverage co-expression of a number of genes; however, the authors make strong statements based on the differential expression of individual genes. There is a risk for spurious associations here given the limited size of the cohorts. It would be important to confirm these results in a small "validation" cohort.

We greatly appreciate this suggestion and accordingly have generated gene expression data for the highlighted genes from another n=9 preserved T cell responses post-DAA therapy (**Extended Data Fig. 6c**). We were able to confirm all but one of the TFs (LMCD1 being the exception) and have changed the figure accordingly.

9. Does the expression of these TF correlate with that of TOX, suggesting a common mechanism of regulation?

Indeed, of these transcription factors and co-factors, ETV1, EOMES and LCMD1 displayed expression patterns correlating with TOX expression, suggesting potential mechanisms of co-regulation (**Extended Data Fig. 6b**).

10. Throughout the paper, the CD8+ T cells specific for an HCV epitope which has escaped (TF-ESC) were a nice intra-donor nice comparative of an exhausted vs non-exhausted HCV-specific CD8+ T cells. However, when the epitope changed to escape, a new set of HCV-specific CD8+ T cells are primed in a highly inflammatory milieu, resulting in some phenotypic and functional differences (Snell et al., Immunity 2018). Could this phenomenon also participate in the difference observed between Tex and Tesc?

This is an intriguing idea, though we have not seen these phenomena in HCV infection. We have followed many patients from acute into chronic infection and the findings were as follows: 1) HCV-specific responses were diminished or even fully disappeared quickly in acute persisting infection, with no indication that novel populations were primed (Cox et al., Hepatology 2005 and unpublished data) 2) The only phenotypic changes we observed were once a response lost its target after viral escape, and these were consistent with what we describe here. Responses targeting fully preserved epitopes have been shown to have rather stable phenotypes from shortly after the acute phase of infection (Kasprowicz et al., J Virol 2009 and unpublished data) 3) We have never observed *de novo* priming of new T cell responses against HCV viral variants, as has

been shown HIV, despite extensive screening (unpublished data). Overall, we have very little indication that there are massive changes or *de novo* occurrences in the repertoire or phenotype of HCV-specific CD8 T cell responses once a patient is in the chronic phase of HCV infection.

MINOR COMMENTS

1. Fig 6e: Is the FDR in red an absolute 0?

Indeed, it was not an absolute 0 and the FDR has been corrected in the figure.

2. In the discussion, the link between the study and checkpoint inhibitor blockade is a little unclear; after all, ICB do not work by removing antigen. Can the authors highlight the importance of their study from other angles? Their results are more broadly relevant indeed.

This is a very valid point and we have added to the discussion of this in the introduction and discussion.

3. Line 295 – 298 could be misleading; it is an accurate statement if the authors are referring to the phenotyping changes between Tex Pre vs Post-DAA only. However, if we take into consideration the transcriptomic data, because there is a number of DEG between Tesc Pre vs Post-DAA, it is hard to associate the differences "overwhelmingly" to TCR stimulation rather than the chronic inflammatory milieu. The wording could be nuanced

We have altered this statement.

4. Typo in line 314 : " […] Tesc [instead of Tex] can still recover by antigen removal alone,in contrast to Tex […]"

This has been corrected.

Reviewer #2:

Extended data figure 2 describes how T cell responses are classified into 'exhausted', or targeting epitopes that have 'partially escaped' or 'fully escaped'. The classification is based on viral diversity in each patient as assessed by next generation sequencing. If I understand the approach correctly, T cell responses are first mapped with a panel of common HCV genotype 1a epitopes (multimer staining) and the sequence of these peptides is then compared to corresponding HCV sequence of the patients. Any difference is defined as mutation (indicated by red letters in panel A). If 100% of the viral sequences from the patient match the sequence of the common HCV genotype 1a epitope, this specific T cell response is classified as 'exhausted'. If 100% of the viral sequences from the patient differ from the sequence of the common HCV genotype 1a epitope, this specific T cell response is classified as 'fully escaped'. If there is sequence heterogeneity in the patient for the epitope of interest, this specific T cell response is defined as 'partially escaped'. In a second part (panel B), T cells stimulated with the respective peptides and IFN-g production after stimulation with variant peptide is compared to IFN-g production after stimulation with common HCV genotype 1a epitope. The relative decrease in IFN-g production is reported.

We greatly appreciate this detailed comment as it helped us to describe the classification of the different responses more clearly in the revised version of the manuscript, as already indicated in our response to reviewer 1. In brief, we used the established genotype 1a epitope sequences for screening of T cell responses in each patient. In case we detected a response, we also obtained the sequence data for this epitope circulating in this patient. If the prototype peptide and the autologous sequence matched, we considered this a fully preserved epitope and thus classified the response as T_{EX}. If we detected a sequence variant, this led to additional assays determining the consequences of the viral variant. Importantly, the classification into full and partial escape was not based on the sequence data, as all but one of the dominant sequences (conserved or variant) in each patient represented close to 100% of viral strains. Rather we performed ICS assays using both the original and the variant epitope peptide sequence and compared the responses. As shown in **Extended Data Fig.2b**, about half of the variant peptides elicited no response or a response less than 10% of the corresponding prototype peptide (lower half of panel **ED Fig.2b**). These were thus classified as full escape. Other peptide variants elicited a response of more than 25% compared to the prototype, but less than 75%. These were considered partial escape. As discussed already in the response to reviewer 1 above, we are very confident that the full escape variants were selected early during infection, based on many previous studies, whereas partially escaped responses might be a mix of escape or priming with a suboptimal sequence. Based on this, we focused on the fully escaped responses for our comparisons. We have edited the manuscript and ED Fig.2 for clarity.

1. Legend to Extended data figure 2: Please add missing word (x): 'Recognition of variant peptide compared to (x).....'

Thank you, this was added.

2. I find it difficult to extrapolate from viral sequence to T cell recognition: Case 115 =(KLVALGINAV) is defined as fully escaped, but there is almost no decrease in IFNg production when variant and wildtype are compared in panel B.

We realize that the figure was not presented in the best way. The grey bars actually mean relative strength of the IFNγ response against the variant compared to the prototype/wild-type sequence. In the case of 115 =(KLVALGINAV) the value is less than 10%, or more than a 10-fold reduction. This has been edited for clarity.

3. It is impossible to know whether patients with partially escaped or fully escaped epitopes actually encountered the wildtype epitope in the acute phase. Alternatively, it is possible that they were infected with a different strain of HCV genotype 1a from the beginning. Without such data, the conclusion 'The idea that duration of antigen stimulation, rather than duration of recovery, is the defining factor for the ability to differentiate into TMEM' should be modified.

We agree that this is the case for partial escape, as stated in the response to reviewer 1 above. And the reviewer would be correct for the full escape epitopes as well if the data were indeed how he or she had interpreted them (that even full escape, for example in case 115 KLVALGINAV, led only to a minimal reduction in IFN-g responsiveness). However, as outlined above, in the case of fully escaped epitopes (on which the conclusion rests), most of these variants completely abrogate the response and thus could not have primed a response during acute infection. We hope that the better explanation of this figure has now clarified this, as for completely abolished responsiveness viral escape is the only explanation consistent with what we know about HCV infection and viral escape. This is further underscored by our and others' studies in acute HCV

infection where exactly the same sequence variations were observed in connection with T cell mediated immune pressure within the first year of infection.

4. NGS data show a mix of HCV sequences for many patients. Do the authors propose that 'partial escape' occurs because the cognate antigen is 'diluted' due to the presence of variant sequences? It is still possible that both sequences are presented to T cells on the same antigen-presenting cell – wouldn't the T cell then receive the full stimulation?

This is a very interesting point. On one hand, none of the fully escaped responses had any trace of the wildtype in the sequence data, and thus no partial TCR stimulation can be assumed. There are two partial escape responses with very small percentages of wild type sequence and also more cases of a mixed population of circulating variants. This could indeed contribute to the more heterogeneous results from the $T_{P\text{-}ESC}$ populations. This is another reason why we focused on the $T_{F\text{-}ESC}$ responses in the analysis and interpretation.

5. The authors analyze sequence diversity at the antigen level (HCV) but not at the TCR level. Different TCRs have different avidity to the respective antigen and it should be considered that change in phenotype and transcriptome after viral clearance is due to specific expansion of cells with specific TCRs within the epitope-specific T cell population. Unfortunately, both flow cytometry analysis and RNAseq analysis are limited to the 'bulk' population of peptide-specific T cells. I think it is important to add CiteSeq combined with TCR analysis. This would add novel information and can be done immediately as the authors have cryopreserved PBMC from the lymphaphereses.

We greatly appreciate this comment and suggestion, as this is a very relevant point indeed. Unfortunately, CiteSeq combined with TCR is currently not readily feasible for the very rare populations we have to analyze. While we would love to do this combined analysis, this was not possible in the limited time to prepare this revision, especially under pandemic conditions. But we have performed TCR studies pre- and post- therapy, displayed in **Fig. 2g,h**, that demonstrate a rather stable clonal composition in all patients studied. Together with the single cell flow data showing pervasive phenotypic changes in pretty much the complete T cell populations post therapy, this establishes that the observations cannot be explained by selective emergence or disappearance of distinct HCV-specific T cell clones.

6. The radar plots show 14 paired samples (pre- versus post HCV clearance) for exhausted CD8 T cells and 8 paired samples for partially exhausted CD8 T cells. Are these from the same patients?

Overall, most of the patients with $T_{F\text{-}ESC}$ had also T_{EX} detected that were included in the analyses (**Fig. 1b**, patients with both red and green dots). Only two patients exclusively had $T_{F\text{-}ESC}$ (Patients 113 and 114). Thus, among the 8 $T_{F\text{-}ESC}$ populations studied in **Fig. 2c**, 6 were coming from the very same patients with the T_{EX} analyzed in **Fig. 2b**.

7. Figure 3E: T-SNE analysis is based on the expression levels of CD38, HLA-DR, PD-1, CD39, TIGIT, CCR7, CD45RA, Integrin-Beta-7 and CD62L, but PCA (Figure 3F) is based on expression level of 37 molecules. How do the data from panel E look in a PCA?

We included this analysis in **Extended Data Fig. 3d**.

Other comments:

8. Figure 1 describes a large panel of EBV, Flu and CMV epitopes, but data are currently only shown for a single patient in Fig. 1D. Please include data on the phenotype of EBV, Flu and CMV-specific T cells in Figure 1C to allow direct comparison with the phenotype of HCV-specific T cells.

We integrated these data in a revised **Fig. 1c**.

9. The methods section contains a sentence that or previously generated T cell lines were used for some experiments. Please explain where in vitro expanded T cell lines (rather than ex vivo studied CD8 T cells) were used.

Overall, all the critical data in this manuscript (phenotypical, functional, and transcriptional analyses) were generated on direct *ex vivo* T cell populations, without any *in vitro* expansion. The one exception are T cell lines that we generated and used in order to test the effect of viral sequence variants on cognate epitope recognition by the T cells (partially or fully escaped) as measured by IFNγ intracellular detection (**Extended data Fig. 2b**). The method section has been clarified accordingly.

Decision Letter, first revision:

Subject: Your manuscript, NI-A29982A

Message: Our ref: NI-A29982A

21st May 2021

Dear Dr. Lauer,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Differentiation of exhausted CD8 T cells after termination of chronic antigen stimulation stops short of achieving functional T cell memory" (NI-A29982A). 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 "Differentiation of exhausted CD8 T cells after termination of chronic antigen stimulation stops short of achieving functional T cell memory". 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.

Cover suggestions

As you prepare your final files we encourage you to consider whether you have any images or illustrations that may be appropriate for use on the cover of Nature Immunology.

Covers should be both aesthetically appealing and scientifically relevant, and should be supplied at the best quality available. Due to the prominence of these images, we do not generally select images featuring faces, children, text, graphs, schematic drawings, or collages on our covers.

We accept TIFF, JPEG, PNG or PSD file formats (a layered PSD file would be ideal), and the image should be at least 300ppi resolution (preferably 600-1200 ppi), in CMYK colour mode.

If your image is selected, we may also use it on the journal website as a banner image, and may need to make artistic alterations to fit our journal style.

Please submit your suggestions, clearly labeled, along with your final files. We'll be in touch if more information is needed.

Nature Immunology has now transitioned to a unified Rights Collection system which will allow our Author Services team to quickly and easily collect the rights and permissions required to publish your work. Approximately 10 days after your paper is formally accepted, you will receive an email in providing you with a link to complete the grant of rights. If your paper is eligible for Open Access, our Author Services team will also be in touch regarding any additional information that may be required to arrange payment for your article.

You will not receive your proofs until the publishing agreement has been received through our system.

Please note that Nature Immunology is a Transformative Journal (TJ). Authors may publish their research with us through the traditional subscription access route or make their paper immediately open access through payment of an article-processing charge (APC). Authors will not be required to make a final decision about access to their article until it has been accepted. Find out more about Transformative Journals.

If you have any questions about costs, Open Access requirements, or our legal forms, please contact ASJournals@springernature.com.

<B>Authors may need to take specific actions to achieve compliance with funder and institutional open access mandates. For submissions from January 2021, if your research is supported by a funder that requires immediate open access (e.g. according to Plan S principles) then you should select the gold OA route, and we will direct you to the compliant route where possible. For authors selecting the subscription publication route our standard licensing terms will need to be accepted, including our self-archiving policies. Those standard licensing terms will supersede any other terms that the author or any third party may assert apply to any version of the manuscript.

Please use the following link for uploading these materials: [REDACTED]

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

Best regards,

Elle Morris
Editorial Assistant
Nature Immunology
Phone: 212 726 9207
Fax: 212 696 9752
E-mail: immunology@us.nature.com

On behalf of

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

The Macmillan Building
4 Crinan Street
Tel: 212-726-9207
Fax: 212-696-9752
z.fehervari@nature.com

Reviewer #1:
Remarks to the Author:
In this revised manuscript, the authors were highly responsive and addressed well the issues raised on the first submission, and the concerns from this reviewer were adequately addressed. The study is well designed, the resulting storyline is coherent and the results strong and interesting. This reviewer has no more issues to raise.

However, findings similar to some of the results presented in this paper have recently been published elsewhere, in particular in the manuscript by Hensel et al., Nat Imm 2021 (after DAA, the TEX move towards a memory-like phenotype and transcriptional profile distinct that memory T cells of resolvers, along with loss of activation and exhaustion-related features; TCR clonotypes are comparable pre and post DAA; TEX shift towards a cytotoxic profile; some exhaustion-related transcription factors do not normalize with DAA).

Therefore, the authors may want to underline more the findings and conclusions that are still unreported and clearly novel in their study:
The rigorous timeline and design of their study, including the long follow up, allow them to investigate and understand the durability of the identified modifications.
Also, the links to the functional capacity of CD8+ T cells is highly relevant.
They show that CD8+ T cells specific for escaped variants have similar functional capacities as HCV-specific CD8+ T cell of resolvers, while the functional capacities of

exhausted HCV-specific CD8+ T cells after DAA remain stunted.
There is some disconnect between "recovery" of some phenotypic features and function - whereas CD127 expression keeps increasing and EOMES, CD39 decreasing past 6 months post-DAA, HCV-specific CD8+ T cells do not recover their functional capacity, underscoring the persisting nature of the "scar".

Reviewer #3:
Remarks to the Author:
The authors have used an elegant approach to study the memory potential of chronically stimulated human CD8 T cells after antigen removal by studying the impact of DAA treatment-induced HCV clearance on HCV-specific CD8 T cell phenotype and function. While comparing HCV-specific CD8 T cells from chronically infected patients after DAA versus those from acute HCV resolvers may give insights about their memory potential, this analysis may be confounded by the possible emergence of escape mutants of HCV that is coupled with variable durations of TCR stimulation among HCV-specific CD8 T cell clones. To overcome this challenge, the authors performed deep viral sequencing to identify the epitope mutations, and categorized HCV-specific CD8 T cells into Tex, Tp-esc, and Tf-esc based on functional assessment of their ability to recognize variant epitopes using in vitro generated T cell lines.

1-Although the authors did TCR-seq of different groups of HCV-specific CD8 T cells, it is not clear how they generated the T cell lines used in (extended Fig. 2b) or how similar T cell line's TCRs to those from ex vivo samples. These TCR sequences may be different from the actual ex vivo TCRs with variable avidity levels to variant viral epitopes. Thus, the definition of partially vs. fully escaped T cells may need to be revisited.
2-In patient 102, C63B epitope-specific CD8 T cells were classified as conserved "exhausted" cells in extended figure 2a (red line), while the same type of cells was defined as partially escaped in panel b of the same figure. In addition, these epitope-specific CD8 T cells were included in the data shown in Figure 5 as an example of Tex. Interestingly, they look less exhausted than other samples. Please clarify whether these cells are Tex or Tp-esc and correct the figure(s) accordingly.
3-Although multi-color flow cytometry and RNA-seq analyses revealed broad phenotypic and transcriptional changes in Tex cells after HCV cure, these analyses are limited and don't reveal insights into the mechanisms underlying the observed lack of function recovery after antigen removal in Tex cells. As the authors pointed out in the discussion section, fixed epigenetic programs may explain the stable dysfunctionality in the recovered Tex cells. Assessing changes in chromatin accessibility and/or global or targeted DNA methylation profiling will potentially reveal the epigenetic programs mediating the loss or recovery of memory potential in Tex or Tesc, respectively. This information will raise the impact of the current findings and give significant insights into the biological mechanisms underlying immune scarring.
4-Comment#10 reviewer 1 is critical. The emergence of escape mutants partially releases the immune pressure on HCV replication and may be coupled with enhanced viral titers, and potentially increases the inflammatory microenvironment. This would result in an increase in the conserved antigen load which plays a key role in enhancing the exhaustion phenotype/programs in Tex cells. The authors need to address this point appropriately with their supporting unpublished data. Are there differences in basal viral titers early (first year after escape) and late during chronic HCV infection or at the pre-DAA treatment phase?

5-Minor comments:
a-Please report in the materials and methods the duration of chronic HCV infection in treated patients as clinically documented to be 10 years for all patients except 1 patient for 19 years. This is an important information to confirm the prolonged TCR stimulation in those patients before DAA treatment relative to acute resolvers.
b-In Fig 7f, several genes/TFs were highlighted as not changing their transcript levels in Tex cells after DAA treatment. Can the authors assess/discuss their functional impact on CD8 T cell biology and memory differentiation?

Author Rebuttal, first revision:

Dear Zoltan, thanks a lot for the update.

Reviewer three makes some interesting points that I wanted to address quickly below as I think they should not require any additional work. I hope this will help you with the decision once the other review comes in. Please let me know if you have any additional questions.

Best

Georg

1-Although the authors did TCR-seq of different groups of HCV-specific CD8 T cells, it is not clear how they generated the T cell lines used in (extended Fig. 2b) or how similar T cell line's TCRs to those from ex vivo samples. These TCR sequences may be different from the actual ex vivo TCRs with variable avidity levels to variant viral epitopes. Thus, the definition of partially vs. fully escaped T cells may need to be revisited.

The reviewer is correct that potentially T cell lines might not perfectly reflect the repertoire of the in vivo population. However, the approach we used is widely accepted (and described in the methods section) and has led to many consistent findings on T cell escape in HCV infection in different published studies, including our own. This consistency is also seen in the present data, as all responses classified into "full escape" by this approach show the identical phenotype that is completely different from exhausted T cells, with "partially escaped" T cell populations occupying the middle ground between full escape and exhaustion. The approach is further supported by a previous publication on early chronic HCV infection, where the comparison was made between escape classification via T cell lines versus direct ex vivo testing (Cox et al., JEM 2005). In this early stage of chronic HCV infection, T cell frequencies are much higher than years later and allow direct ex vivo studies, and there was no difference in the results. Overall, it seems extremely unlikely that any additional investigation would lead to re- classification of responses, especially those labeled full escape, on which our conclusions rest. Finally, we would also like to point out that our effort to classify escape responses is extensive and thorough and beyond that of most papers in the literature, where sequence variations often are directly equated with viral escape, without any further experimental support (including the NI paper from the Thimme group).

2-In patient 102, C63B epitope-specific CD8 T cells were classified as conserved "exhausted" cells in extended figure 2a (red line), while the same type of cells was defined as partially escaped in panel b of the same figure. In addition, these epitope-specific CD8 T cells were included in the data shown in Figure 5 as an example of Tex. Interestingly, they look less exhausted than other samples. Please clarify whether these cells are Tex or Tp-esc and correct the figure(s) accordingly.

The reviewer is correct and clearly had a very careful look at the data. This response is the only one with a variant sequence that has only a minor impact on T cell recognition, with the variant response almost 75% of that of the prototype peptide. We define escape to have a reduction to 50% or less (with full escape to 10% or less) and thus we classified this as an exhausted response. This needs to be updated in figure S2B by adjusting the orange frame marking the partially escaped responses to exclude 102 c63b.

3-Although multi-color flow cytometry and RNA-seq analyses revealed broad phenotypic and transcriptional changes in Tex cells after HCV cure, these analyses are limited and don't reveal insights into the mechanisms underlying the observed lack of function recovery after antigen removal in Tex cells. As the authors pointed out in the discussion section, fixed epigenetic programs may explain the stable dysfunctionality in the recovered Tex cells. Assessing changes in chromatin accessibility and/or global or targeted DNA methylation profiling will potentially reveal the epigenetic programs mediating the loss or recovery of memory potential in Tex or Tesc, respectively. This information will raise the impact of the current findings and give significant insights into the biological mechanisms underlying immune scarring.

We certainly agree with the reviewer that, while our extensive analysis is unusually broad and deep, including identification of some potential new key transcriptional regulators of the exhausted phenotype, the data cannot fully establish all potential mechanisms underlying the continued dysfunctional phenotype. As you know, the specific question raised here is very thoroughly addressed in the Haining paper, which is why we are convinced that the joint publication of these manuscripts (plus the Wherry paper) would create an unusually complete and compelling story.

4-Comment#10 reviewer 1 is critical. The emergence of escape mutants partially releases the immune pressure on HCV replication and may be coupled with enhanced viral titers, and potentially increases the inflammatory microenvironment. This would result in an increase in the conserved antigen load which plays a key role in enhancing the exhaustion phenotype/programs in Tex cells. The authors need to address this point appropriately with their supporting unpublished data. Are there differences in basal

viral titers early (first year after escape) and late during chronic HCV infection or at the pre-DAA treatment phase?

This is an interesting point that we already discussed extensively in our response to reviewer 1. Regarding the additional point about increased viral loads and inflammation after viral escape, we would like to point out that there is sufficient published literature demonstrating that viral loads are relatively stable in the chronic phase and can be both higher and lower than during the first year of infection. Liver enzymes as signs of liver inflammation are typically much lower after the first year of infection, even with persistent viremia. Overall, this is in agreement with our and other groups' data that maximal levels of exhaustion are typically already reached in the first year of infection. That differences in viral load are not the key factor for the irreversibility of exhaustion is further supported by the fact that the patients in our study had pre-treatment viral loads over a wide range from 10,000 to 10,000,000 IU/ml, with uniform results.

--

Georg Lauer MD PhD

Response to Review of NI-A29982A

We thank the reviewers for their positive comments regarding this "well designed study" using an "elegant approach" with "strong and interesting results" that is "a significant contribution to the field" and "fosters our understanding of persistent virus-specific CD8 T cell impairment and residual exhaustion after therapeutic antigen removal in humans." We have addressed the remaining points raised by the reviewers in a point-by-point fashion below.

Reviewers' Comments:

Reviewer #1:

Therefore, the authors may want to underline more the findings and conclusions that are still unreported and clearly novel in their study: The rigorous timeline and design of their study, including the long follow up, allow them to investigate and understand the durability of the identified modifications. Also, the links to the functional capacity of CD8+ T cells is highly relevant. They show that CD8+ T cells specific for escaped variants have similar functional capacities as HCV-specific CD8+ T cell of resolvers, while the functional capacities of exhausted HCV-specific CD8+ T cells after DAA remain stunted. There is some disconnect between "recovery" of some phenotypic features and function - whereas CD127 expression keeps increasing and EOMES, CD39 decreasing past 6 months post-DAA, HCV-specific CD8+ T cells do not recover their functional capacity, underscoring the persisting nature of the "scar".

We greatly appreciate the reviewer's confirmation that our study adds significant insights beyond the paper that was recently published by Hensel et al. As suggested, we have now highlighted the specific opportunities offered by our study being designed specifically for immunological investigation, the addition of direct ex vivo functional studies, and the additional information provided by a detailed definition of different degrees of viral escape.

Reviewer #3:

1-Although the authors did TCR-seq of different groups of HCV-specific CD8 T cells, it is not clear how they generated the T cell lines used in (extended Fig. 2b) or how similar T cell line's TCRs to those from ex vivo samples. These TCR sequences may be different from the actual ex vivo TCRs with variable avidity levels to variant viral epitopes. Thus, the definition of partially vs. fully escaped T cells may need to be revisited.

The reviewer is correct that potentially T cell lines might not perfectly reflect the repertoire of the *in vivo* population. However, the approach we used is widely accepted (and described in the methods section) and has led to many consistent findings on T cell escape in HCV infection in different published studies, including our own. This consistency is also seen in the present data, as all responses classified into "full escape" by this approach show the identical phenotype that is completely different from exhausted T cells, with "partially escaped" T cell populations occupying the middle ground between full escape and exhaustion. The approach is further supported by a previous publication on early chronic HCV infection, where the comparison was made between escape classification via T cell lines versus direct ex vivo testing (Cox et al., JEM 2005). In this early stage of chronic HCV infection, T cell frequencies are much higher than years later and allow direct ex vivo studies, and there was no difference in the results. Overall, it seems extremely unlikely that any additional investigation would lead to re- classification of responses,

especially those labeled full escape, on which our conclusions rest. Finally, we would also like to point out that our effort to classify escape responses is extensive and thorough and beyond that of most papers in the literature, where sequence variations often are directly equated with viral escape, without any further experimental support.

2-In patient 102, C63B epitope-specific CD8 T cells were classified as conserved "exhausted" cells in extended figure 2a (red line), while the same type of cells was defined as partially escaped in panel b of the same figure. In addition, these epitope-specific CD8 T cells were included in the data shown in Figure 5 as an example of Tex. Interestingly, they look less exhausted than other samples. Please clarify whether these cells are Tex or Tp-esc and correct the figure(s) accordingly.

The reviewer is correct and clearly had a very careful look at the data. This response is the only one with a variant sequence that has only a minor impact on T cell recognition, with the variant response almost 75% of that of the prototype peptide. We define escape to have a reduction to 50% or less (with full escape to 10% or less) and thus we classified this as an exhausted response. This has been updated in figure S2B by adjusting the orange frame marking the partially escaped responses to exclude 102 C63B.

3-Although multi-color flow cytometry and RNA-seq analyses revealed broad phenotypic and transcriptional changes in Tex cells after HCV cure, these analyses are limited and don't reveal insights into the mechanisms underlying the observed lack of function recovery after antigen removal in Tex cells. As the authors pointed out in the discussion section, fixed epigenetic programs may explain the stable dysfunctionality in the recovered Tex cells. Assessing changes in chromatin accessibility and/or global or targeted DNA methylation profiling will potentially reveal the epigenetic programs mediating the loss or recovery of memory potential in Tex or Tesc, respectively. This information will raise the impact of the current findings and give significant insights into the biological mechanisms underlying immune scarring.

We certainly agree with the reviewer that, while our extensive analysis is unusually broad and deep, including identification of some potential new key transcriptional regulators of the exhausted phenotype, the data cannot fully establish all potential mechanisms underlying the continued dysfunctional phenotype. As you know, the specific question raised here is very thoroughly addressed in the companion paper by Drs Haining and Sen, which is why we are convinced that the joint publication of these manuscripts will create an unusually complete and compelling story.

4-Comment#10 reviewer 1 is critical. The emergence of escape mutants partially releases the immune pressure on HCV replication and may be coupled with enhanced viral titers, and potentially increases the inflammatory microenvironment. This would result in an increase in the conserved antigen load which plays a key role in enhancing the exhaustion phenotype/programs in Tex cells. The authors need to address this point appropriately with their supporting unpublished data. Are there differences in basal viral titers early (first year after escape) and late during chronic HCV infection or at the pre-DAA treatment phase?

This is an interesting point that we already discussed extensively in our response to reviewer 1, who was satisfied with our answer. Regarding the additional point about increased viral loads and inflammation after viral escape, we would like to point out that there is sufficient published literature demonstrating that viral loads are relatively stable in the chronic phase of HCV infection

and can be both higher and lower than during the first year of infection. Liver enzymes as signs of liver inflammation are typically much lower after acute infection, even with persistent viremia. Overall, this is in agreement with our and other groups' data that maximal levels of exhaustion are typically already reached in the first year of infection. That differences in viral load are not the key factor for the irreversibility of exhaustion is further supported by the fact that the patients in our study had pre-treatment viral loads over a wide range from 10,000 to 10,000,000 IU/ml, with uniform results.

5-Minor comments:

a-Please report in the materials and methods the duration of chronic HCV infection in treated patients as clinically documented to be 10 years for all patients except 1 patient for 19 years. This is an important information to confirm the prolonged TCR stimulation in those patients before DAA treatment relative to acute resolvers.

This has been added.

b-In Fig 7f, several genes/TFs were highlighted as not changing their transcript levels in Tex cells after DAA treatment. Can the authors assess/discuss their functional impact on CD8 T cell biology and memory differentiation?

This is a great suggestion, but apart from the recently established role of TOX for T cell exhaustion, these genes have not been described in great detail in this context. This makes it very speculative to discuss their specific impact on T cells, but we will further investigate these molecules.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A29982B

Message: In reply please quote: NI-A29982B

Dear Dr. Lauer,

I am delighted to accept your manuscript entitled "Differentiation of exhausted CD8 T cells after termination of chronic antigen stimulation stops short of achieving functional T cell memory" for publication in an upcoming issue of Nature Immunology.

The manuscript will now be copy-edited and prepared for the printer. Please check your calendar: if you will be unavailable to check the galley for some portion of the next month, we need the contact information of whom will be making corrections in your stead. When you receive your galleys, please examine them carefully to ensure that we have not inadvertently altered the sense of your text.

Acceptance is conditional on the data in the manuscript not being published elsewhere, or announced in the print or electronic media, until the embargo/publication date. These restrictions are not intended to deter you from presenting your data at academic meetings and conferences, but any enquiries from the media about papers not yet scheduled for publication should be referred to us.

Please note that Nature Immunology is a Transformative Journal (TJ). Authors may publish their research with us through the traditional subscription access route or make their paper immediately open access through payment of an article-processing charge (APC). Authors will not be required to make a final decision about access to their article until it has been accepted. Find out more about Transformative Journals.

<B>Authors may need to take specific actions to achieve compliance with funder and institutional open access mandates. For submissions from January 2021, if your research is supported by a funder that requires immediate open access (e.g. according to Plan S principles) then you should select the gold OA route, and we will direct you to the compliant route where possible. For authors selecting the subscription publication route our standard licensing terms will need to be accepted, including our self-archiving policies. Those standard licensing terms will supersede any other terms that the author or any third party may assert apply to any version of the manuscript.

In approximately 10 business days 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.

You will not receive your proofs until the publishing agreement has been received through our system.

If you have any questions about our publishing options, costs, Open Access requirements, or our legal forms, please contact ASJournals@springernature.com

Once your manuscript is typeset and you have completed the appropriate grant of rights, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately. Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details.

Your paper will be published online soon after we receive your corrections and will appear in print in the next available issue. Content is published online weekly on Mondays and Thursdays, and the embargo is set at 16:00 London time (GMT)/11:00 am US Eastern time (EST) on the day of publication. Now is the time to inform your Public Relations or Press Office about your paper, as they might be interested in promoting its publication. This will allow them time to prepare an accurate and satisfactory press release. Include your manuscript tracking number (NI-A29982B) and the name of the journal, which they will need when they contact our office.

About one week before your paper is published online, we shall be distributing a press release to news organizations worldwide, which may very well include details of your work. We are happy for your institution or funding agency to prepare its own press release, but it must mention the embargo date and Nature Immunology. Our Press Office will contact you closer to the time of publication, but if you or your Press Office have any enquiries in the meantime, please contact press@nature.com.

Also, if you have any spectacular or outstanding figures or graphics associated with your manuscript - though not necessarily included with your submission - we'd be delighted to consider them as candidates for our cover. Simply send an electronic version (accompanied by a hard copy) to us with a possible cover caption enclosed.

To assist our authors in disseminating their research to the broader community, our SharedIt initiative provides you with a unique shareable link that will allow anyone (with or without a subscription) to read the published article. Recipients of the link with a subscription will also be able to download and print the PDF.

As soon as your article is published, you will receive an automated email with your shareable link.

You can now use a single sign-on for all your accounts, view the status of all your manuscript submissions and reviews, access usage statistics for your published articles and download a record of your refereeing activity for the Nature journals.

If you have not already done so, we strongly recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. Protocol Exchange is an open online resource that allows researchers to share their detailed experimental know-how. All uploaded protocols are made freely available, assigned DOIs for ease of citation and fully searchable through nature.com. Protocols can be linked to any publications in which they are used and will be linked to from your article. You can also establish a dedicated page to collect all your lab Protocols. By uploading your Protocols to Protocol Exchange, you are enabling researchers to more readily reproduce or adapt the methodology you use, as well

as increasing the visibility of your protocols and papers. Upload your Protocols at www.nature.com/protocolexchange/. Further information can be found at www.nature.com/protocolexchange/about .

Please note that we encourage the authors to self-archive their manuscript (the accepted version before copy editing) in their institutional repository, and in their funders' archives, six months after publication. Nature Research recognizes the efforts of funding bodies to increase access of the research they fund, and strongly encourages authors to participate in such efforts. For information about our editorial policy, including license agreement and author copyright, please visit www.nature.com/ni/about/ed_policies/index.html

An online order form for reprints of your paper is available at https://www.nature.com/reprints/author-reprints.html. Please let your coauthors and your institutions' public affairs office know that they are also welcome to order reprints by this method.

Sincerely,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

The Macmillan Building
4 Crinan Street
Tel: 212-726-9207
Fax: 212-696-9752
z.fehervari@nature.com