nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: Two subsets of stem-like CD8$^+$ memory T cell progenitors with distinct fate commitments in 6 humans
Corresponding author name(s): Enrico Lugli

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A29689A

Message: 27th May 2020

Dear Dr Lugli,
Thank you for the our discussion the other day your response to the Referee issues - this was helpful for our decision-making.
We are interested in the possibility of publishing your study in Nature Immunology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Note, I've marked-up your response letter with some comments to help guide your revision (see attached). Please highlight all changes in the manuscript text file in Microsoft Word format.

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.

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.

* 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.

* Please include a revised version of any required reporting checklist. It will be available to referees to aid in their evaluation of the manuscript goes back for peer review. They are available here:

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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,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

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Referee expertise:

Referee #1: T cell differentiation, exhaustion

Referee #2: T cell differentiation, exhaustion

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The study be Galletti use a sophisticated state of the art strategy of combining scRNAseq and high dimensional flow cytometry profiling to resolve the heterogeneity of human memory T cells. They make the surprising discovery that the human memory population contains a subset that is transcriptionally, functionally and epigenetically very similar to the progenitors of T cell exhaustion found in chronic infection and tumors. Moreover, the authors show that these T cells bear a unique TCR repertoire. This points out that the cells develop independently from other memory populations and that they recognize a unique antigen-repertoire. The data is therefore an elegant demonstration that sheds new light on the phenomenon of T cell exhaustion. It points out that the phenotype we normally associate with rare or very specific diseases (certain chronic infections and cancer) are nothing uncommon. Instead, cells with features of T cell exhaustion seem to comprise of a significant fraction of the normal T cell repertoire.

At the same time, I found another point very important and interesting, which the authors in my opinion did not feature prominently enough. It is widely believed that Tscm constitute a superior quality of memory T cell than classical Tcm. This distinction is supported by clear transcriptional difference between Tscm and Tcm. However, Galletti at al. show that Tcm are contaminated by GzmK expressing cells and that upon their removal, Tscm and Tcm look alike. This really questions that concept of Tscm and I think this important correction should be more prominently featured in the manuscript.

Overall, I think the manuscript it timely and has significant and highly important messages. I have only two major points and several minor points to be addressed upon revision.

Major points:

- I need to admit that I was slightly overwhelmed by the phenotyping data that are extensively discussed on the first 5 pages of the MS. I mean I fully appreciate the efforts of the authors to illustrate their strategy, but one needs to go through quite a bit of information of which the significance is not always very clear. Restructuring and streamlining these sections would presumably be appreciated by a broader reader audience.

- I also think that the abstract and title are slightly generic and need to better feature the key conclusion of the study.

- One key limitation is that the authors want to make broadly applicable conclusion about the exitance of this exhausted precursor population among healthy individuals. What remains slightly unclear is how broadly applicable this conclusion is. So far, too little information is provided about the donor cells taken from Fig 1a. How many patients were

used? Age? Sex? Random selection of patients? I think some more evidence or information is needed to generalize these findings.

Minor points:

- The authors talk about "early differentiated memory T cell pool" and "long-lived memory T cell pool". It would be helpful to better explain the use to the terminology.

- The line "Overall, these data suggest transcriptional heterogeneity in the 22 early differentiated memory T cell pool (Fig. 1, B and D, and Supplementary Table 1)" is unclear

- The authors introduce a lot of surface markers and then there are some inconsistencyies with the markers they are using to label a specific population.

- Page 3 - Line 6: it is unclear what they mean when they state "memory populations are thought to be organized in a developmental hierarchy analogous to somatic tissues"

- Figure 2: the gating strategy and populations definition are unclear and difficult to follow. Maybe a comprehensive gating tree might help visualize these different populations.

- Figure 2D: Is there also some cytokine expression data? IFNg, TNFa?

- Page 10 line 13 "produced cytokines more frequently" – suggests that these cells produce cytokines more commonly or often with respect to time. I think the authors mean at a higher frequency?

- Figure 3J: was there any phenotypic flow analysis on these T cells after the experiment?

- Fig 4j: is there any later time points for this data? It looks like the RLU of the Tscm is catching up to the Tmex at later time points.

- Figure 5: It could be informative to compare single cell data from Tmex and Tscm to data that is already in the literature from exhausted progenitors (in the setting of tumor or chronic infection) and Tscm (in acute infection).

Reviewer #2:
Remarks to the Author:
Galletti, et al., "Two subsets of stem-like CD8+ memory T cell progenitors with distinct fate commitments in humans"

Galletti and colleagues describe studies of novel human memory CD8+ T cell subsets that possess differential characteristics within classically defined central memory CD8+ T cells, as defined by CCR7 and CD45RA/RO. Through a large scRNAseq (10X genomics) approach, they identify a set of 10 bulk clusters of unique CD8+ T cell signatures, and define the transcriptomic properties of each. They develop a flow based panel employing some of the core differential signatures and use this to identify within central memory T cells the presence of two subsets that have a differential exhaustion state. One population carries signatures related to stem cell memory T cells (Tscm), originally defined by the

senior author. The other population is more reminiscent of an exhausted phenotype, although it is perhaps not as exhausted as a classical Tex cell. However this new cell type, termed Tmex, does carry some classical signatures of exhaustion. The authors go into great detail to define the epigenetics and function of this subset through a variety of assays including adoptive transfer into humanized mice. They end by showing that antiviral CD8+ T cells (Rota, flu, CMV, EBV) can have this phenotype, and that it is enriched in EBV-specific cells.

Overall the work is of very high quality, the manuscript is very well written, and the figures look fantastic. The main concern I have here is the overall message. While maybe this subset has not been defined to this degree, it has been known for a very long time that Tcm cells can express inhibitory receptors, such as PD-1. It is therefore not entirely surprising that the individual cells bearing these inhibitory receptors would indeed have a differential transcriptomic and epigenetic signature. EBV specific cells are known also to be highly skewed towards a PD-1 expressing Tcm phenotype (R. Ahmed, JI). However, whether one can appropriately conclude that these are exhausted seems premature. EBV is very well controlled by CD8+ T cells in the vast majority of individuals, and perhaps the phenotypic nature of these PD-1 expressing Tcm-like cells reflects their lymphoid tissue trafficking ability thus enabling them to access the infected B cell populations. Altogether, though I find the work exceptionally high quality, the overall study seems relatively incremental towards redefining current models of CD8+ T cell differentiation.

Additional Specific Points:
1) Nomenclature: The authors propose a number of new abbreviations for their cell types, including Tmex and Tstem. I found this somewhat confusing in the context of all the other abbreviations used in the field. The Tstem population is a combination of Tscm and non-PD-1/TIGIT expressing Tcm. While I understand the rationale here to simplify, previous work by the senior author went to great lengths to define Tscm as an entirely unique subset, with greater stem-like properties compared to Tcm. This makes the data hard to interpret, as the Tstem population is a heterogenous population. The Tmex abbreviation, which originates from the Tex subset designation, is somewhat tricky as well, as it is part of the Tcm population. In addition, it is possible that the Tmex name might be offensive to some cultures, thus recommend changing this name.
2) Can the authors speculate on the increased CD69 upregulation apparent after stimulation? What function would this have? Would this enforce tissue residency, perhaps in response to antigen as cells travel through tissue?
3) Do the Tmex cells express CD28? The majority of Tcm do, but this is not clearly specified. This is relevant because of the stimulation protocols and the discrepancy stated between PMA/IM and anti-CD3/28 stimulation. If Tmex do not express CD28, one might expect them to not function as well without co-stimulation. Did the authors explore use of anti-CD49d, commonly used as an alternative co-stimulatory strategy for CD8s?
4) In Figure 3I, J, the authors show that by d28 circulating Tmex vs Tstem roughly equilibrate. Do the cells maintain their phenotype in vivo? In addition, the spleen does not seem to equilibrate. Do the authors have an explanation for this? The spleen is a heavily blood penetrated organ, and largely should reflect the composition of the blood. Can the authors differentiate CD8s in lymphoid follicles/white pulp from CD8s in the red pulp? Perhaps in these mice by d28 there are high numbers of follicles in the spleen that are better populated by the Tstem, rather than the Tmex? Or do the Tmex cells traffic into white pulp as well (presumably so due to presence of CCR7)?
5) In figure 5, HIV+ subjects are included- why? And were HIV-specific responses also examined?

6) For the TCR analysis, this was all done with bulk cells. Is there any evidence that the same clonotype can be found within Tstem and Tmex cells? The clonotyping data shown was all performed, seemingly, on bulk sorted cells. This is not sufficient to prove or disprove the model proposed in 5G. It is also possible that the Tmex are a subset of Tstem that simply has been driven further in response to chronic antigen. This is unfortunately nearly impossible to understand in humans, the presence of shared clonality within an antigen specific population could have occurred early yielding different subsets in a bifurcated model, or late in a linear model. However, as it stands now, the potential model in 5G is not supported by the data. Examining the clonotypes of antigen-specific Tstem vs. Tmex in the same individual would shed light on this, but still be difficult to interpret into a model.

Author Rebuttal to Initial comments

nature research

Manuscript No. NI-A29689A

"Two subsets of stem-like CD8+ memory T cell progenitors with distinct fate commitments in humans"

Galletti et al.
Corresponding author: Enrico Lugli, Humanitas Clinical and Research Center, Milan, Italy

Point-by-point response to Reviewers' comments

Reviewer #1

(Remarks to the Author)
The study be Galletti use a sophisticated state of the art strategy of combining scRNAseq and high dimensional flow cytometry profiling to resolve the heterogeneity of human memory T cells. They make the surprising discovery that the human memory population contains a subset that is transcriptionally, functionally and epigenetically very similar to the progenitors of T cell exhaustion found in chronic infection and tumors. Moreover, the authors show that these T cells bear a unique TCR repertoire. This points out that the cells develop independently from other memory populations and that they recognize a unique antigen-repertoire. The data is therefore an elegant demonstration that sheds new light on the phenomenon of T cell exhaustion. It points out that the phenotype we normally associate with rare or very specific diseases (certain chronic infections and cancer) are nothing uncommon. Instead, cells with features of T cell exhaustion seem to comprise of a significant fraction of the normal T cell repertoire.

At the same time, I found another point very important and interesting, which the authors in my opinion did not feature prominently enough. It is widely believed that Tscm constitute a superior quality of memory T cell than classical Tcm. This distinction is supported by clear transcriptional difference between Tscm and Tcm. However, Galletti at al. show that Tcm are contaminated by GzmK expressing cells and that upon their removal, Tscm and Tcm look alike. This really questions that concept of Tscm and I think this important correction should be more prominently featured in the manuscript.

Overall, I think the manuscript it timely and has significant and highly important messages. I have only two major points and several minor points to be addressed upon revision.

Authors. We emphasized these important concepts in the revised version of the Abstract, Introduction and Discussion. Concerning the presence of T cells with exhaustion-like features in healthy individuals, please also see our response to Reviewer 2 below. Reviewer 2 suggests that Tmex have exhaustion traits but are probably not as exhausted as those T cells isolated from tumors or from chronic viral infections. We agree with this statement, thus, throughout the paper, we now refer to Tmex as dysfunctional/exhaustion-like cells. This in also line with a recent discussion with other leaders in the field (Blank et al, Nat Rev Immunol. 2019 Nov;19(11):665-674).
We agree with the Reviewer that clear transcriptional differences were identified between Tscm and Tcm in our paper by Gattinoni*, Lugli* et al, Nat Med. 2011 Sep 18;17(10):1290-7, **Figure 3d**. The surprising finding is that the majority of those genes now delineate the Tstem and Tmex subsets, specifically *TOX*, *TGFBR3*, *HNRNPLL*, *DUSP4*, *PRRL5*, *IGF1R* (all with q-val<0.05; **Supplementary Table 4**), *PHACTR2*, *SLFN11*, *HMGB3*, *FCER1G* (all with p-val <0.05; not shown). This is now briefly mentioned on page 9. These data demonstrate that the difference previously ascribed to Tscm and Tcm is mainly due to a contamination of the transcriptionally

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distinct Tmex population. We would like to stress that our new findings do not question the concept of Tscm, rather define an improved strategy to expand the more functional stem-like T cell pool, with important practical consequences for translational applications.

Major points:

- I need to admit that I was slightly overwhelmed by the phenotyping data that are extensively discussed on the first 5 pages of the MS. I mean I fully appreciate the efforts of the authors to illustrate their strategy, but one needs to go through quite a bit of information of which the significance is not always very clear. Restructuring and streamlining these sections would presumably be appreciated by a broader reader audience.

Authors. We agree with the Reviewer that much information is included to describe the complex heterogeneity of the memory T cell compartment in humans. We modified the manuscript to focus on the most essential information that is important for the main message of the paper.

- I also think that the abstract and title are slightly generic and need to better feature the key conclusion of the study.

Authors. We thank the reviewer for suggesting these changes. We believe that the title reflects the main message. We slightly restructured the abstract to include suggestions mentioned above.

- One key limitation is that the authors want to make broadly applicable conclusion about the existance of this exhausted precursor population among healthy individuals. What remains slightly unclear is how broadly applicable this conclusion is. So far, too little information is provided about the donor cells taken from Fig 1a. How many patients were used? Age? Sex? Random selection of patients? I think some more evidence or information is needed to generalize these findings.

Authors. We thank the Reviewer for raising this aspect. We have expanded the Introduction and the Discussion to strengthen the concept that our improved identification of functional stem-like T cells (Tstem) overcomes the paucity of Tscm cells obtained ex vivo, and that we provide a defined molecular identity that should be obtained in manufacturing protocols to improve adoptive T cell transfer immunotherapy.
We have included more information on the selection of donors. In the scRNAseq experiment, we analyzed a cohort of 4 healthy females, aged 24-39 years old. These findings are then confirmed by high-dimensional flow cytometry in a larger cohort of 120 samples from blood bank donors (males and females), aged 18-65 years old and selected randomly, or cancer patients donating PB and tumor-free lymph node or lung samples (n=6 each). A detailed description of the these samples is now included in **Supplementary Table 1**.

Minor points:

- The authors talk about “early differentiated memory T cell pool” and “long-lived memory T cell pool”. It would be helpful to better explain the use to the terminology.

Authors. Both terms refer to the same concept. We unified the terminology (“early differentiated”) in the revised version of the manuscript.

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- The line "Overall, these data suggest transcriptional heterogeneity in the early differentiated memory T cell pool (Fig. 1, B and D, and Supplementary Table 1)" is unclear

Authors. We removed this statement in the revised version of the manuscript.

- The authors introduce a lot of surface markers and then there are some inconsistencies with the markers they are using to label a specific population.

Authors. We believe the Reviewer has possibly missed the gating strategy used to define and isolate the different memory T cell populations (**Supplementary Fig. 1**). We have edited the text for ease of understanding. It is unclear if additional changes are requested.

- Page 3 - Line 6: it is unclear what they mean when they state "memory populations are thought to be organized in a developmental hierarchy analogous to somatic tissues"

Authors. We thank the Reviewer for raising this aspect. We removed our statement on somatic tissues as this is irrelevant for the message of the paper.

- Figure 2: the gating strategy and populations definition are unclear and difficult to follow. Maybe a comprehensive gating tree might help visualize these different populations.

Authors. We apologize that this was not clear. Please see our response above on this regard: a gating tree identifying the T cell subsets isolated in our study has been originally included in **Supplementary Fig. 1**.

- Figure 2D: Is there also some cytokine expression data? IFNg, TNFa?

Authors. We did not perform analysis of effector cytokine production by FACS following activation of T cell subsets with anti-CD3/28 as in Figure 2D. In particular, we thought there was no need to investigate cytokine production capacity of Tscm and Tcm depleted of the PD-1+ TIGIT+ (Tmex) population, as these subsets were highly similar in terms of gene expression profiles ex vivo and proliferation capacity in vitro. Instead, data on cytokine production capacity of Tmex vs Tstem following activation in vitro with anti-CD3/28 for 4 days can be extrapolated from transcriptional profiling in **Figure 4h** and **Supplementary Table 4**. While we could not find differences in IFNg and TNF, we instead found significant increased expression of *IL2* (in line with functional data ex vivo shown in **Figure 3**), *CSF2* (encoding GM-CSF) and *GNLY* mRNA in Tstem compared to Tmex (**Figure 4h**). In a similar experiment, Tstem produced more GZMB compared to Tmex, as measured by flow cytometry (**Figure 4e**). Overall, these data highlight an increased functional/killing capacity of Tstem vs. Tmex, also confirmed in vivo in an adoptive T cell transfer model of leukemia (**Figure 4j**).

- Page 10 line 13 "produced cytokines more frequently" – suggests that these cells produce cytokines more commonly or often with respect to time. I think the authors mean at a higher frequency?

Authors. The Reviewer is correct. We corrected the statement.

- Figure 3J: was there any phenotypic flow analysis on these T cells after the experiment?

9

Authors. We thank Reviewer 1, together with Reviewer 2, for this question. We have performed extensive phenotypic analysis of the T cells transferred to NSG mice, and we did not find major

differences as far as their distribution among memory phenotypes is concerned. In our hands, T cells always acquire an activated, CCR7– CD45RO+ effector phenotype in these mice, irrespectively of the phenotype of origin (**Extended Figure 14** in Gattinoni*, Lugli* et al, Nat Med, 2011 Sep18;17(10):1290-7). Therefore, this model is inadequate to test T cell self-renewal in vivo because of the excessive immune activation occurring in a xenogeneic environment. The long-term repopulation experiment (informing on long-term persistence) performed in **Figure 3** and showing that Tstem are superior to Tmex, is an alternative approach to overcome this limitation. The phenotypic profiles of the transferred cells are not mentioned in the manuscript because they are not instrumental for the overall message of the paper. We will be happy to include such data in further Supplementary Figures should the Reviewer think this is important.

- Fig 4j: is there any later time points for this data? It looks like the RLU of the Tscm is catching up to the Tmex at later time points.

Authors. Unfortunately, longer follow-up is not available. As stated in Methods, we stopped follow-up and sacrificed mice to collect organs when RLU values $\geq 10^6$ were observed in more than 75% of mice in one of the treated groups.

- Figure 5: It could be informative to compare single cell data from Tmex and Tscm to data that is already in the literature from exhausted progenitors (in the setting of tumor or chronic infection) and Tscm (in acute infection).

Authors. To avoid possible technical bias resulting from merging data from independent studies (such as subsets from acute and chronic infections), we used scRNAseq of melanoma TILs from Sade-Feldman et al., Cell. 2019 Jan 10;176(1-2):404 and identified signatures of *CCR7+ GZMK–* memory-like and of *CCR7+ GZMK+ TCF7+* progenitor-exhausted CD8+ T cells (clusters G10 and G5, respectively, from **Figure 1c** and Supplementary Table 1). We have previously shown that progenitor exhausted/stem-like CD8+ T cells from lung and melanoma tumors also express *GZMK* (detected by scRNAseq) and the inhibitory receptors PD-1 and TIGIT (detected by flow cytometry), and share identity with TCF-1+ TIM3- progenitors from chronic LCMV infection (Brummelman J et al, J Exp Med. 2018 Oct 1;215(10):2520-2535). The *CCR7+ GZMK–* memory-like gene signature overlaps with the Tstem gene signature, while the *CCR7+ GZMK+ TCF7+* progenitor-exhausted gene signature overlaps with the Tmex gene signature (p-val= 4.7e-19 and 7.1e-28, respectively; hypergeometric test). These results, along with the lists of overlapping genes, are shown in **Supplementary Table 4** and mentioned on page 10 of the manuscript. Please note that we already compared the Tstem signature to that of antigen-specific Tscm induced by vaccination, and showed that these have a shared identity (FDR=0.002, **Figure 5a**).

Reviewer #2

(Remarks to the Author)
Galletti, et al., "Two subsets of stem-like CD8+ memory T cell progenitors with distinct fate commitments in humans"

Galletti and colleagues describe studies of novel human memory CD8+ T cell subsets that possess differential characteristics within classically defined central memory CD8+ T cells, as defined by CCR7 and CD45RA/RO. Through a large scRNAseq (10X genomics) approach, they identify a set of 10 bulk clusters of unique CD8+ T cell signatures, and define the transcriptomic properties of each. They develop a flow based panel employing some of the core differential signatures and use this to identify within central memory T cells the presence of two subsets that have a differential exhaustion state. One population carries signatures related to stem cell memory T cells (Tscm), originally defined by the senior author. The other population is more reminiscent of an exhausted phenotype, although it is perhaps not as exhausted as a classical Tex cell. However this new cell type, termed Tmex, does carry some classical signatures of exhaustion. The authors go into great detail to define the
epigenetics and function of this subset through a variety of assays including adoptive transfer into humanized mice. They end by showing that antiviral CD8+ T cells (Rota, flu, CMV, EBV) can have this phenotype, and that it is enriched in EBV-specific cells.

Overall the work is of very high quality, the manuscript is very well written, and the figures look fantastic. The main concern I have here is the overall message. While maybe this subset has not been defined to this degree, it has been known for a very long time that Tcm cells can express inhibitory receptors, such as PD-1. It is therefore not entirely surprising that the individual cells bearing these inhibitory receptors would indeed have a differential transcriptomic and epigenetic signature. EBV specific cells are known also to be highly skewed towards a PD-1 expressing Tcm phenotype (R. Ahmed, JI). However, whether one can appropriately conclude that these are exhausted seems premature. EBV is very well controlled by CD8+ T cells in the vast majority of individuals, and perhaps the phenotypic nature of these PD-1 expressing Tcm-like cells reflects their lymphoid tissue trafficking ability thus enabling them to access the infected B cell populations. Altogether, though I find the work exceptionally high quality, the overall study seems relatively incremental towards redefining current models of CD8+ T cell differentiation.

Additional Specific Points:
1) Nomenclature: The authors propose a number of new abbreviations for their cell types, including Tmex and Tstem. I found this somewhat confusing in the context of all the other abbreviations used in the field. The Tstem population is a combination of Tscm and non-PD-1/TIGIT expressing Tcm. While I understand the rationale here to simplify, previous work by the senior author went to great lengths to define Tscm as an entirely unique subset, with greater stem-like properties compared to Tcm. This makes the data hard to interpret, as the Tstem population is a heterogenous population. The Tmex abbreviation, which originates from the Tex subset designation, is somewhat tricky as well, as it is part of the Tcm population. In addition, it is possible that the Tmex name might be offensive to some cultures, thus recommend changing this name.

Authors. We thank the Reviewer for the appreciation of our work and for raising specific points. The Reviewer claims that it is known that Tcm can express PD-1 and it is not entirely surprising that these cells are transcriptionally and epigenetically distinct. S/he also claims that antigen-specific cells can control EBV in vivo despite high PD-1 expression. 11

The central questions, in the opinion of the authors, are if stem-like T cells with dysfunctional/exhaustion-like features can be identified also in healthy individuals, have reduced functionality and have a distinct fate commitment compared to conventional, stem-like memory cells. Our results demonstrate that Tscm and Tcm-phenotype cells expressing PD-1 (Tmex) are functionally inferior at multiple levels compared to those lacking these inhibitory receptors (the Tstem). Most importantly, epigenetic modifications at exhaustion and effector-related loci specifically found in Tmex are inherited by the progeny, thereby suggesting that epigenetic modifications may constitute a major barrier to reprogramming towards a functional state, as described for Tex in chronic infections (Pauken KE, Science. 2016 Dec 2;354(6316):1160-1165). The additional finding that the TCR repertoire of Tstem and Tmex is largely (~95%) non-overlapping (**Figure 5e**) indicates that Tstem and Tmex are not mere distinct functional states, rather they recognize a relatively unique antigen repertoire.

Although not addressed directly in our work, we agree with the Reviewer that Tmex are not as exhausted as a classical Tex cells. Throughout the paper, we now refer to these cells as dysfunctional/exhausted-like, as some authors describe Tex as cells with unconventional/divergent functions rather than with diminished functions (Blank et al, Nat Rev Immunol. 2019 Nov;19(11):665-674). However, please note that TCF-1+ progenitors in tumors or chronic viral infections maintain some degree of functionality and persistence compared to terminally differentiated Tex, and for this reason they are generally referred to as stem-like (reviewed in Lugli et al., Trends Immunol, 2020; Kallies et al. Nat Rev Immunol. 2020 Feb;20(2):128-136; Held W, Sci Transl Med. 2019 Oct 23;11(515):eaay6863). It is thus inherently difficult to adopt a nomenclature that fully describes these characteristics. We thank the Reviewer for suggesting that the term Tmex might not be appropriate. We propose to change the name to "progenitors of exhausted T cells", or "Tpex", as recently proposed in a consensus review with leaders in the field (Blank et al, Nat Rev Immunol. 2019 Nov;19(11):665-674) and by Kallies and colleagues (Kallies et al. Nat Rev Immunol. 2020 Feb;20(2):128-136).

The Reviewer also asserts that the Tstem described in this study is heterogeneous, because comprising Tscm and non-PD-1/TIGIT-expressing Tcm. However, our data support the opposite conclusion, i.e., that Tscm and Tcm depleted of the Tmex are instead the same population, both transcriptionally and functionally (**Figure 1** and **2**). Rather, Tcm, that are generally referred to as a homogeneous population in humans, preferentially include Tmex that are transcriptionally, epigenetically and functionally distinct. Notably, those genes previously found differentially expressed by Tscm and Tcm in our original report (see **Figure 3d**, Gattinoni*, Lugli* et al, Nat Med. 2011 Sep 18;17(10):1290-7) are indeed signature genes of the Tmex population in this study (please also see our detailed response to Reviewer 1 in this regard). Thus, our data do not question the concept of Tscm, rather define an improved strategy to expand the stem-like T cell pool with superior functional capacity.

In conclusion, the discovery of a shared identity of Tscm and Tcm cells, and of a novel subset of stem-like T cells hard-wired to its dysfunctional/exhaustion-like state in healthy humans are unprecedented aspects of human T cell biology that shed light on the mechanisms leading to long term protective immunity.

2) Can the authors speculate on the increased CD69 upregulation apparent after stimulation? What function would this have? Would this enforce tissue residency, perhaps in response to antigen as cells travel through tissue?

Authors. CD69 has been historically used as an activation marker of in vitro activated T cells. It is difficult to speculate about the tissue resident differentiation potential of Tstem and Tmex only on the basis of this marker. In **Figure 1h-i**, we have shown that these subsets are mostly found in LNs

where they are virtually negative for the tissue residency markers CD69 and CD103. A similar profile is found in the bone marrow. Unfortunately, the virtual absence of Tstem and Tmex in the lung (<1% of the total memory CD8+ T cells) does not allow a reliable evaluation of the expression of CD69 and CD103 in these cells. Therefore, we would avoid to speculate in this regard in the absence of definitive data.

3) Do the Tmex cells express CD28? The majority of Tcm do, but this is not clearly specified. This is relevant because of the stimulation protocols and the discrepancy stated between PMA/IM and anti-CD3/28 stimulation. If Tmex do not express CD28, one might expect them to not function as well without co-stimulation. Did the authors explore use of anti-CD49d, commonly used as an alternative co-stimulatory strategy for CD8s?

Authors. Both Tstem and Tmex express CD28 at equivalent levels (please see UMAP analysis in **Figure 1h** and our statement on page 7 line 15. We did not explore CD49d, or other costimulatory receptors. However, we did not see differences in *ITGA4* (encoding CD49d) mRNA or mRNAs of other costimulatory molecules in RNAseq data, both ex vivo (**Figure 2c**) and after stimulation in vitro (**Figure 4h**).

4) In Figure 3I, J, the authors show that by d28 circulating Tmex vs Tstem roughly equilibrate. Do the cells maintain their phenotype in vivo? In addition, the spleen does not seem to equilibrate. Do the authors have an explanation for this? The spleen is a heavily blood penetrated organ, and largely should reflect the composition of the blood. Can the authors differentiate CD8s in lymphoid follicles/white pulp from CD8s in the red pulp? Perhaps in these mice by d28 there are high numbers of follicles in the spleen that are better populated by the Tstem, rather than the Tmex? Or do the Tmex cells traffic into white pulp as well (presumably so due to presence of CCR7)?

Authors. Please see response to Reviewer 1 above regarding the phenotype of the transferred T cell subsets in NSG mice. Although we could not follow these mice longer than 28 days because of possible xenoGVHD reactions, dynamics in the blood suggest that Tstem and Tmex tend to equilibrate not only because of the late expansion of the Tmex, but also because of the contraction of Tstem, likely due to their rapid proliferation in vivo. However, the number of human T cells recovered from the spleen largely exceeded that from the blood (10 to 20 times depending on the transferred cell type), thus stressing the importance of the difference found between Tstem and Tmex numbers in the spleen at sacrifice. Differentiating between CD8 in the white vs red pulp in the NSG model is probably not optimal in the opinion of the authors. In fact, NSG mice lack T and B cells, preventing the formation of murine lymphoid follicles. The infusion of human PBMCs in NSG mice results in poor engraftment of B cells in these mice, generating poorly organized germinal centers (Shultz L.D. et al., Nat Rev Immunol, 2012, Nov;12(11):786-98). Accordingly, the vast majority (>95%) of cells in the spleens at sacrifice were CD3+, thus suggesting that lymphoid follicles are largely under-represented. Finally, CCR7 is rapidly downregulated following adoptive transfer due to immune activation, thus possibly questioning its role in trafficking.

5) In figure 5, HIV+ subjects are included- why? And were HIV-specific responses also examined?

Authors. Analysis of HIV– and HIV+ individuals revealed overlapping phenotypic profiles of antigen-specific CD8+ T cells, thus results were combined. Unfortunately, we do not have data on HIV-specific T cell responses that we can rapidly include in a revised version of the manuscript.

13

In any case, a predominant PD-1+ TIGIT+ (Tmex) rather than a PD-1– TIGIT– (Tstem) profile is expected in HIV-specific CD8+ T cells from these individuals, on the basis of previous data (Chew GM, et al, PLoS Pathog. 2016 Jan 7;12(1):e1005349).

6) For the TCR analysis, this was all done with bulk cells. Is there any evidence that the same clonotype can be found within Tstem and Tmex cells? The clonotyping data shown was all performed, seemingly, on bulk sorted cells. This is not sufficient to prove or disprove the model proposed in 5G. It is also possible that the Tmex are a subset of Tstem that simply has been driven further in response to chronic antigen. This is unfortunately nearly impossible to understand in humans, the presence of shared clonality within an antigen specific population could have occurred early yielding different subsets in a bifurcated model, or late in a linear model. However, as it stands now, the potential model in 5G is not supported by the data. Examining the clonotypes of antigen-specific Tstem vs. Tmex in the same individual would shed light on this, but still be difficult to interpret into a model.

Authors. We understand the Reviewer's concern on the differentiation model proposed in **Figure 5g**. Only a minor proportion of clonotypes found in Tstem is also found in Tmex (~5% of the top 3000 clones, compared to ~17% of Tstem vs Tem and ~30% of Tmex vs Tem; **Figure 5e** and **5f**). Thus, although possible, identification of shared clonotypes between Tstem and Tmex is an infrequent event. We agree with the Reviewer that Tmex can ideally form from differentiated Tstem following chronic exposure to antigen at high dose, as previously outlined in mice chronically infected with LCMV by Ahmed and colleagues (West E, Immunity. 2011 Aug 26;35(2):285-98). We edited our model and the Discussion to include this aspect. Although our data cannot formally exclude it, the Tmex→Tstem transition is instead unlikely to occur, as Tmex-specific epigenetic modifications are inherited in the progeny following stimulation (**Figure 4** and **Supplementary Figure 4**). This is in line with previous findings showing the phenotypic (Utzschneider DT, Nat Immunol. 2013 Jun;14(6):603-10) and epigenetic (Pauken KE, Science. 2016 Dec 2;354(6316):1160-1165; Ghoneim HE et al, Cell. 2017 Jun 29;170(1):142-157.e19) stability of the exhausted CD8+ T cell lineage.

Decision Letter, first revision:

Subject: Nature Immunology - NI-A29689B pre-edit

Message: Our ref: NI-A29689B

30th Jul 2020

Dear Dr. Lugli,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Two subsets of stem-like CD8+ memory T cell progenitors with distinct fate commitments in humans" (NI-A29689B). Please follow the instructions provided here and in the attached files, as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

When you upload your final materials, please include a point-by-point response to the points below. We won't be able to proceed further without this detailed response.

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3. As a guideline, Articles allow up to 50 references in the main text. An additional 20 references can be included in the Online Methods. Only papers that have been published or accepted by a named publication or recognized preprint server should be in the numbered list. Published conference abstracts, numbered patents and research data sets that have been assigned a digital object identifier may be included in the reference list.

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5. Genes must be clearly distinguished from gene products (e.g., "gene Abc encodes a kinase," not "gene Abc is a kinase"). For genes, provide database-approved official

symbols (e.g., NCBI Gene, http://www.ncbi.nlm.nih.gov/gene) for the relevant species the first time each is mentioned; gene aliases may be used thereafter. Italicize gene symbols and functionally defined locus symbols; do not use italics for proteins, noncoding gene products and spelled-out gene names.

Figures and Tables:

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8. All relevant figures must have defined error bars.

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Other
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We ask that you aim to return your revised paper within 7 days. If you have any further questions, please feel free to contact me.

Best regards,

Zoltan Fehervari, Ph.D.
Senior Editor
Nature Immunology

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Reviewer #1:
Remarks to the Author:
This is an excellent manuscript that deserves to be published without delay. The presented data widely broadens our conceptual understanding of the phenomenon of T cell exhaustion. All of my question were fully answered and congratulation to the authors.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A29689C

Message: In reply please quote: NI-A29689C

Dear Dr. Lugli,

I am delighted to accept your manuscript entitled "Two subsets of stem-like CD8+ memory T cell progenitors with distinct fate commitments in humans" 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.

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Sincerely,

Zoltan Fehervari, Ph.D.
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