nature portfolio

Peer Review File

IL-4 impairs skin-resident memory CD8+ T cell formation

Corresponding Author: Dr Shannon Bromley

Version 0:

Decision Letter:

31st Aug 2021

Dear Dr. Bromley,

Your Article, "IL-4 impairs skin-resident memory CD8+ T cell formation" has now been seen by 2 referees. While the work is of potential interest, the reviewers have raised substantial concerns that must be addressed. While we find your work of interest, the reviewers have raised substantial concerns that must be addressed. As such, we cannot accept the current version of the manuscript for publication, but would be happy to consider a revised version that addresses these concerns, as long as novelty is not compromised in the interim.

If you decide to resubmit a revised manuscript, at resubmission please include a point-by-point “Response to referees” detailing how you have addressed each referee comment (please specify page and figure number). This response will be sent back to the referees along with the revised manuscript.

In addition, please 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.

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We would hope to receive the revised manuscript 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.

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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,

Ioana Visan, Ph.D.
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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript Mora-Buch and colleagues report that IL-4 diminishes the formation of CD8+ tissue-resident memory T cells by interfering with CD103 expression. The initial observation that IL-4 negatively affects CD103 expression stems from a set of straight forward in vitro experiments and the authors then try to address in vivo relevance before moving back to an in vitro set up to define the potential mechanism of IL-4-mediated inhibition of Trm formation. Briefly, the authors suggest that IL-4 acts directly on CD8 T cells, which leads to reduced expression of TGFbRII (with concurrent TGFb signals).
I enjoyed reading this manuscript, which was well written and easy to follow.
Overall, the presented findings are novel and potentially significant, but there are concerns regarding the in vivo biological relevance. However, these concerns could be addressed by the authors.

Comments re. physiological relevance and conclusions:

Fig. 2: the 4get mice indicate the potential to make IL-4 (transcript present), but that is not equivalent to available IL-4 protein (shown by Mohrs and colleagues - PMID: 16226507); the KN2 mice made by Mohrs et al. would address when and where IL-4 protein is made, which would be more informative to understand when/where IL-4 could affect CD8 Trm formation.
The authors assessed CD8 T cell numbers and phenotype in IL-4Ra-/- mice (and Stat6-/- mice) and the data are consistent with their hypothesis, but these data are also tricky to interpret since IL-4Ra is expressed by several difference cell types (there could be direct and indirect effects).

Fig. 3: The authors activate OT-I T cells in vitro with or without IL-4 and then adoptively transfer these cells into DNFB treated mice; these experiments clearly show that IL-4 treated OT-I T cells have a distinct CD103 phenotype and also distribution pattern (compared to untreated OT-Is), but also raise the question of physiological relevance (when would this happen under physiologic conditions?). Importantly, IL-4 can promote naïve CD8 T cell proliferation (PMID: 11447288) – does the in vitro culture with IL-4 change other CD8 T cell properties in this experimental set-up (differences in initial take after adoptive transfer, memory phenotypes on day 28, etc.)?
If the authors can calculate absolute numbers in skin and spleen and plot these numbers instead of ratios, it'd would be make for much more intuitive plots and stronger conclusions.

Fig. 4: The 2 sets of experiments in this figure are critical as they address the in vivo relevance of the observations from the first 3 figures; the authors transfer WT and IL-4Ra-/- OT-I T cells into IL-4Ra-/- recipients followed by an OVA tattoo priming and MC903 Tx to elicit atopic dermatitis. It is unclear to me why the recipient mice are not simply WT mice? As mentioned for the adoptive transfer expt. shown in Fig. 3, calculating absolute numbers instead of ratios would substantially strengthen the conclusions to determine the decrease in cell numbers in the skin (and potential differences in the spleen between WT and IL-4Ra-/- OT-I T cells).
The authors also try to assess a potential difference in the ability to protect using the same initial set-up as in panel a, but then followed by depletion of peripheral CD8 T cells (but not CD8 Trm) and a skin HSV-OVA challenge. It would make more sense to deplete using congenic markers (to deplete peripheral OT-Is only) instead of rendering the animals lymphopenic prior to infection. It also difficult to assess the biological significance of the pfu load without a naïve control group and an undepleted control group. Panel f shows the compiled data from 2 experiments – for the IL-4Ra-/- OT-Is ("red dots"):
are the 5 pfu low datapoints from one of the experiments and the 5 pfu high data points from the 2nd experiment? If that's the case, it'd suggest that a difference was observed in one experiment, but not the technical repeat.
Finally, is there a correlation between peripheral depletion efficiency and pfu load?

Fig. 5. As stated above, since IL-4 can induce naïve T cell proliferation, is the decrease in TGFbRII expression that is

observed in context of TGFb availability dependent on the number of cell divisions (if the cells were CFSE or CTV labeled, does the decrease occur after 1, 2, 3, etc. cell divisions and is it transient or sustained?)

If the authors can make a stronger case that IL-4 signals reduce the Trm numbers in the skin and to the point that protection is affected, this could be a very exciting study. The paper would also benefit from some additional insight regarding the TGFbRII downregulation (see my comments re. Fig. 5 – when does it occur, how long does it last).

Reviewer #2:
Remarks to the Author:
Mora-Buch and co-workers describe an inhibitory effect of IL-4 on TGFb-mediated induction of CD103 expression in activated CD8+ T cells that they link to impaired generation of CD103+ resident memory CD8+ T cells in skin. While previous studies have focussed on tissue environmental factors that promote the generation of such cells, very little is known about factors that counteract their generation and maintenance. As such, their study is topical and of great interest. As outlined below however, there are several areas of concern that significantly dampen my enthusiasm for the presented work.

Major comments:

* Fig 2a: TGFb-mediated CD103 induction in WT cells in this graph is considerably less efficient compared to what is shown in Fig.1b/c/d and 3b (all with small error bars). In fact, Il4ra-/- and Stat6-/- cells in Fig.2a show the same level of CD103 induction as the WT cells in Fig.1. Does this reflect different culture conditions between the experiments? Could the authors please clarify?

* Fig 2d: As the plots are pre-gated on CD8+ T cells, it would be a lot more informative to show CD44 versus CD103 expression, particularly since gating on CD44hi versus low can be a little arbitrary. This is also important given that IL-4 can impact thymic development of CD8+ T cells (work from Hogquist lab and others) and it is the thymus-egressing naïve CD44low cells that express intermediate levels of CD103.

* Addition of IL-4 to activated CD8+ T cells can lead to profound and long-lasting downregulation of CD8 surface expression (see e.g. Erard F et al. Science 1993; Kienzle N et al. J Immunol 2005; and other several papers). Somewhat surprisingly, this is not evident from the FACS plots in Fig.1B and Fig. 3B, but I am assuming these are pre-gated on CD8+ events anyway? Regardless, can the authors rule out that this phenomenon, that is loss or reduction of CD8 expression after IL-4 stimulation, impacted on their enumeration of CD8+ T cells from tissues – particularly if results are shown as CD8-pregated events (also Fig. 3J/L and elsewhere)?

* For experiments in Fig.4, could the authors please provide the rationale as to why they chose Il4ra-/- hosts instead of WT mice? The fact that IL4RA is not only missing in the transferred CD8 T cells but also in other immune cells (that may assist TRM generation) appears to unnecessarily complicate the interpretation of the results. Have they done similar experiments in WT mice?

* Fig. 4f: While the authors conclude that the modestly improved protection in Il4ra-/- mice is explained by a reduction in TRM numbers, an alternative or additional explanation could be that IL-4 inhibits or fine tunes antiviral CD8 effector functions. This is well documented in the literature (e.g. Erard-F et al, Science 1993; Sharma-DP et al, J Virol 1996; and many others) and complicates conclusions drawn from this experiment. Have they tested effector functions such as killing or cytokine production in these conditions? Also, what is the level of protection relative to non-transferred mice given that the OVAp tattoo will also generate endogenous OVA-specific TRM cells that should mediate protection?

* Fig.5A: IL-4 promotes downregulation of TGFR only in conjunction with TGFb stimulation. That is interesting but unfortunately this phenomenon remains unexplored.

* The authors repeatedly state that “expression of the transcription factor, Eomes is downregulated by TGFb-signaling” and seem to build some of their mechanistic framework around this assumption. However, their own data in Fig.5C/D as well as some of the literature they cite (Nath et al, PLoS One 2019) do not appear to support this. On another note, the effects on Eomes are unrelated to TGFR modulation as IL-4 alone induces Eomes upregulation but not TGFR downregulation. Overall, it remains largely unclear mechanistically as to how IL-4 may interfere with TGFb-mediated induction of CD103 in vitro or TRM cells in vivo.

Minor comments:
* In many panels the authors show normalised data and sometimes even ratios thereof which makes it rather difficult to gauge the extent and robustness of the experimental differences. For instance, what does “normalised to recovery” mean (Fig.3/4)? Percentages? Numbers? Or “normalised to TGF condition” – for which genotype/s? Where feasible and applicable, the authors should also provide more easily interpretable values, e.g. % CD103+ cells, cell numbers etc.

* Fig. 2c: Because the symbols overlay the bar graphs, I could not figure out the extent of the differences between the conditions. Can the authors please provide numbers as well, or enlarge the graphs?

* Fig 2c: Is there a reduction in total CD8+ T cells as well, or is this specific for CD103-expressing CD8+ T cells?

Version 1:

Decision Letter:

Dear Dr. Bromley,

Thank you for your response to the reviewers' comments on your manuscript "IL-4 impairs skin-resident memory CD8+ T cell formation". We are happy to inform you that if you revise your manuscript appropriately in response to the referees' comments and our editorial requirements your manuscript should be publishable in Nature Immunology.

Please revise your manuscript to include the bar graphs for mean and median and also for Eomes+ cells only as an Extended Data Figure (please note that Supplementary figures are now Extended Data Figures). In addition, please clearly discuss in the Discussion the experimental setups in which an effect of TGFb on Eomes expression was reported, and how were these set ups are different from your experiments and results. At resubmission, please include a point-by-point response to the referees' comments, noting the pages and lines where the changes can be found in the revision. Please highlight the changes in the revised manuscript as well. As a note, articles have a maximum of 4000 words, 8 Figures and 10 Extended data figures. If your current manuscript does not fit these requirements, please revise accordingly.

We are trying to improve the quality and transparency of methods and statistics reporting in our papers (please see our editorial in the May 2013 issue). Please update the Life Sciences Reporting Summary, and supplements if applicable, with any information relevant to any new experiments and upload it (as a Related Manuscript File) along with the files for your revision. If nothing in the checklist has changed, please upload the current version again.

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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 "IL-4 impairs skin-resident memory CD8+ T cell formation". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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We hope to receive your revised manuscript in 10 days, by 10th Apr 2025. Please let us know if circumstances will delay submission beyond this time. If you have any questions please do not hesitate to contact me.

Sincerely,

Ioana Staicu, Ph.D.
Senior Editor
Nature Immunology

Tel: 212-726-9207
Fax: 212-696-9752
www.nature.com/ni

Reviewer #1 (Remarks to the Author):

The authors addressed all my previous comments/concerns. I think this is a really nice, thorough and comprehensive story!

Just one last, small comment - in re to the Eomes expression observed here vs in previous studies. There is no consistency in the field when it comes to assessing the MFI of a population. Some groups compare the whole cell population (incl. cells that do not express the protein of interest), while other groups only examine MFI expression between the protein-expressing cells (gate on the + cells, then assess MFI). Also, some groups use the mean, some the geometric mean while others use the median fluorescence. It'd be good if the authors could make sure that the difference in Eomes expression (refs 19, 20) is not simply due to differences in MFI assessment.

Reviewer #2 (Remarks to the Author):

The authors should be commented for generating and adding a large body of new data to their study. They have addressed all concerns raised in the initial review.

Minor comment:
The title of Fig.2 appears to be wrong as it states: Fig. 2. IL-4 decreases CD103 expression and cutaneous CD8+ TRM numbers during homeostasis. There is no data in this Figure/study to support this statement.

Version 2:

Decision Letter:

Our ref: NI-A32631B

11th Apr 2025

Dear Dr. Bromley,

Thank you for submitting your revised manuscript "IL-4 impairs skin-resident memory CD8+ T cell formation" (NI-A32631B). We are happy to inform you that if you revise your manuscript appropriately according to our editorial requirements, your manuscript should be publishable in Nature Immunology.

I will now pre-edit the current version of your paper. We will also perform detailed checks on your paper and will send you a

checklist detailing our editorial and formatting requirements in about two weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

If you had not uploaded a Word file for the current version of the manuscript, we will need one before beginning the editing process; please email that to immunology@us.nature.com at your earliest convenience.

In the meantime, please deposit all omic and code data into public repositories so that the accession codes are readily available to be added in the revised manuscript. We cannot accept the paper without the codes. In addition, the ORCID of ALL corresponding authors needs to be linked to their Nature account (this frequently causes delays at acceptance). Should you have any query or comments about ORCID, please do not hesitate to contact our editorial assistant at immunology@us.nature.com.

Thank you again for your interest in Nature Immunology. Please do not hesitate to contact me if you have any questions.

Sincerely,

Ioana Staicu, Ph.D.
Senior Editor
Nature Immunology

Tel: 212-726-9207
Fax: 212-696-9752
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We would like to thank the reviewers for their thoughtful suggestions. We have substantially revised the manuscript to include additional data, as suggested, which can be found in 31 new data panels (Fig. 2c, 2d, 2e, 2f, 2h, 2i, 2j, 2k, 4e, 4g, 5c, 5d, 5e, 5h, 5i, 5j and Extended Data Fig. 2a, 2b, 4c, 4f, 4g, 5l, 5m, 7a-g, 8). Additionally, we have re-formatted some figures, as suggested, to improve the clarity of the data presentation (Fig. 1e, 3e, 3g, 3k, 3m and Extended Data Fig. 1c, 3g, 3i, 3j, 4a, 4b, 4d, 4e, 6). We think that these revisions have substantially improved our study, and we hope that you will now find our manuscript suitable for publication. Because changes were substantial, we did not highlight changes in the manuscript. However, our point-by-point response is detailed below.

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

In this manuscript Mora-Buch and colleagues report that IL-4 diminishes the formation of CD8+ tissue-resident memory T cells by interfering with CD103 expression. The initial observation that IL-4 negatively affects CD103 expression stems from a set of straight forward in vitro experiments and the authors then try to address in vivo relevance before moving back to an in vitro set up to define the potential mechanism of IL-4-mediated inhibition of Trm formation. Briefly, the authors suggest that IL-4 acts directly on CD8 T cells, which leads to reduced expression of TGFbRII (with concurrent TGFb signals). I enjoyed reading this manuscript, which was well written and easy to follow. Overall, the presented findings are novel and potentially significant, but there are concerns regarding the in vivo biological relevance. However, these concerns could be addressed by the authors.

Comments re. physiological relevance and conclusions:

Fig. 2: the 4get mice indicate the potential to make IL-4 (transcript present), but that is not equivalent to available IL-4 protein (shown by Mohrs and colleagues - PMID: 16226507); the KN2 mice made by Mohrs et al. would address when and where IL-4 protein is made, which would be more informative to understand when/where IL-4 could affect CD8 Trm formation. The authors assessed CD8 T cell numbers and phenotype in IL-4Ra-/- mice (and Stat6-/- mice) and the data are consistent with their hypothesis, but these data are also tricky to interpret sinceIL-4Ra is expressed by several difference cell types (there could be direct and indirect effects).

To determine IL-4 protein production, we imported KN2 mice and analyzed hCD2 expression by flow cytometric analysis of skin and lymph nodes at homeostasis (Fig. 2c, 2h; page 7 line 178-181, page 8 line 217-219). While we consistently observed a population of lymph node $CD4^+$ T cells that express hCD2 in naïve KN2 mice, few $hCD2^+$ $CD4^+$ T cells were recovered from skin, and numbers were quite variable between mice. Following skin painting with MC903, expression of hCD2 by $CD4^+$ T cells in both lymph node and skin are increased (Extended Data Fig. 5l, m; page 11 line 302-303).

We have analyzed cutaneous $CD8^+$ T_{RM} numbers and phenotype in the skin and lymph nodes of *CD8*^{cre} *Il4ra*$^{fl/-}$ and littermate control naïve mice. We observed differences in the frequencies of $CD44^-$ $CD103^+$ $CD8^+$ T cells in the brachial lymph node and spleen of these mice (Fig. 2e, f; page 7 line 190-193). However, we could not detect significant differences in the numbers or phenotype of $CD8^+$ $CD44^{hi}$ T cells recovered from the skin of these mice at homeostasis (Fig. 2j, k; page 8-9 line 219-223). These data correlate with our finding that $CD8^+$ T cells isolated from $IL4^{-/-}$ OT-I mice do not have an advantage compared to WT OT-I T cells in cutaneous $CD8^+$ T_{RM} formation (Extended Data Fig. 3i; page 8 line 204-208), suggesting that although expression of IL-4 in the lymph node at homeostasis may influence naïve $CD8^+$ T cell phenotype, it does not appear to affect subsequent $CD8^+$ T_{RM} formation in the skin where these cells may be exposed to TGF-β, but little IL-4. We thank the reviewers for bringing up this important point, and we have revised the manuscript based on our new results.

Fig. 3: The authors activate OT-I T cells in vitro with or without IL-4 and then adoptively transfer these cells into DNFB treated mice; these experiments clearly show that IL-4 treated OT-I T cells have a distinct CD103 phenotype and also distribution pattern (compared to untreated OT-Is), but also raise the question of physiological relevance (when would this happen under physiologic conditions?). Importantly, IL-4 can promote naïve CD8 T cell proliferation (PMID: 11447288) – does the in vitro culture with IL-4 change other CD8 T cell properties in this experimental set-up(differences in initial take after adoptive transfer, memory phenotypes on day 28, etc.)?If the authors can calculate absolute numbers in skin and spleen and plot these numbers instead of ratios, it'd would be make for much more intuitive plots and stronger conclusions.

MC903 painting, a model for atopic dermatitis, induces IL-4 expression in the draining lymph node as well as in the skin (Extended Data Fig. 5l, m; page 11 line 301-303). Therefore, we hypothesized that cutaneous Th2-type inflammation might not only act in the skin to affect cutaneous $CD8^+$ T_{RM} formation, but perhaps also within the lymph node during $CD8^+$ T cell activation to affect subsequent $CD8^+$ T_{RM} formation. Because the MC903 model induces IL-4 expression not only in the lymph node, but also in the skin, we decided instead to activate the $CD8^+$ T cells *in vitro* in the presence or absence of IL-4, and then adoptively co-transfer these cells into congenic recipient mice that we painted with DNFB to induce non-specific inflammation, $CD8^+$ T cell recruitment into the skin and cutaneous $CD8^+$ T_{RM} formation. In this manner we could expose naïve $CD8^+$ T cells to IL-4 during their initial activation in the lymph node to determine its consequence on subsequent $CD8^+$ T_{RM} formation independent of exposure to IL-4 within an atopic dermatitis lesion.

We now discuss the possibility that T cell activation in the presence of IL-4 might have physiological relevance if $CD8^+$ T cell activation occurs in a lymph node that drains the skin of an atopic dermatitis flare (page 16, line 435-444). This situation could be significant if the same lymph node drains, for example, a site of HSV recrudescence, a site of vaccination (e.g. vaccinia virus), etc. T cell activation in the presence of IL-4 might affect $CD8^+$ T_{RM} formation within the skin at the site of atopic dermatitis, and perhaps even within skin sites that are distinct from the atopic dermatitis flare but still drain to the same lymph node.

We have examined initial take after adoptive transfer, and we did not observe significant differences in the numbers of T cells activated in the presence or absence of IL-4 recovered from either the bLN or skin on day 2 (Extended Data Fig. 4c; page 10 line271-274). We also compared memory phenotype on day 28. While there was a significant difference in the gMFI of CD103 expression by $CD8^+$ T cells recovered from the skin (Fig. 3k; page 10 line 280-282), we did not detect differences in expression of CD49a, CD69 or CD127 (Extended Data Fig. 4f, g; page 10-11 line 276-280).

We have now revised Figure 3g and 3m to show ratios of WT : WT + IL-4 $CD8^+$ T cells recovered from each tissue in the hope that this presentation will be more intuitive. Additionally, we have

now added Extended Data Fig. 4a-4e to show absolute numbers of transferred T cells recovered from each tissue. (Page 10 line 252-255; page 10 line 274-276).

Fig. 4: The 2 sets of experiments in this figure are critical as they address the in vivo relevance of the observations from the first 3 figures; the authors transfer WT and IL-4Ra-/- OT-I T cells into IL-4Ra-/- recipients followed by an OVA tattoo priming and MC903 Tx to elicit atopic dermatitis. It is unclear to me why the recipient mice are not simply WT mice? As mentioned for the adoptive transfer expt. shown in Fig. 3, calculating absolute numbers instead of ratios would substantially strengthen the conclusions to determine the decrease in cell numbers in the skin (and potential differences in the spleen between WT and IL-4Ra-/- OT-I T cells). The authors also try to assess a potential difference in the ability to protect using the same initial set-up as in panel a, but then followed by depletion of peripheral CD8 T cells (but not CD8 Trm) and a skin HSV-OVA challenge. It would make more sense to deplete using congenic markers (to deplete peripheral OT-Is only) instead of rendering the animals lymphopenic prior to infection. It also difficult to assess the biological significance of the pfu load without a naïve control group and an undepleted control group. Panel f shows the compiled data from 2 experiments – for the IL-4Ra-/- OT-Is ("red dots"):are the 5 pfu low datapoints from one of the experiments and the 5 pfu high data points from the2nd experiment? If that's the case, it'd suggest that a difference was observed in one experiment,but not the technical repeat. Finally, is there a correlation between peripheral depletion efficiency and pfu load?

We used *Il4ra*$^{-/-}$ recipient mice because we reasoned that any differences in the numbers or phenotype of CD8$^+$ WT vs. *Il4ra*$^{-/-}$ OT-I T cells recovered from the skin of *Il4ra*$^{-/-}$ mice would be independent of any indirect effects of IL-4/IL-13 on host cells. Because the *Il4ra*$^{-/-}$ OT-I T cells formed CD8$^+$ T_{RM}, but numbers of WT CD8$^+$ T_{RM} were reduced, we reasoned that the effect of IL-4/IL-13 on CD8$^+$ T_{RM} formation/persistence was due to direct effects on the WT CD8$^+$ T cells (Page 12 line 320-322). Of note, we have now found that while both WT and *Il4ra*$^{-/-}$ adoptively transferred CD8$^+$ T cells persist in *Il4ra*$^{-/-}$ recipient mice, adoptively transferred *Il4ra*$^{-/-}$ T cells fail to reliably persist well in the tissues (spleen, lymph node, or skin) of recipient WT mice. We are not certain of the reason for this observation, but it may be due to the fact that these *Il4ra*$^{-/-}$ mice were backcrossed onto C57Bl/6. These results are similar to what we have seen for other back-crossed mouse lines; Thy1.1$^+$ T cells transferred into Thy1.2$^+$ mice do not reliably persist long-term whereas adoptively transferred Thy1.2$^+$ T cells fare better in Thy1.1$^+$ mice. An alternative possibility for this observation is that because IL-4 can promote the survival of CD8$^+$ T cells, perhaps there is a compensatory cytokine produced in *Il4ra*$^{-/-}$ mice that promotes the survival of adoptively transferred *Il4ra*$^{-/-}$ (and WT) CD8$^+$ T cells. Of note, however, WT CD8$^+$ T cells persist in WT mice even when they are treated with anti-IL-4 neutralizing antibody (see below).

To gain more confidence that our findings using *Il4ra*$^{-/-}$ recipient mice translate to WT mice, we have now performed similar experiments in which we adoptively transfer WT OTI T cells into WT congenic recipient mice, tattoo the mice with OVA plasmid, and then treat the mice with either a neutralizing antibody directed against IL-4 or with an isotype control antibody during MC903 painting (page 12 line 323-328). We find that mice treated with the anti-IL-4 antibody have increased numbers of cutaneous CD8$^+$ T_{RM} compared to mice treated with isotype control antibody at a memory time point, suggesting that the effect is IL-4-dependent (Extended Data Fig. 7a-c; page 12-13 line 328-331).

Additionally, we have compared CD8$^+$ T_{RM} formation in *Il4ra* conditional knockout mice. We immunized mice with poly i:c and ovalbumin in order to activate endogenous OVA-specific CD8$^+$ T cells. Then, we tattoo-immunized mice with OVA plasmid to recruit them into the skin for CD8$^+$ T_{RM} formation. Finally, we used MC903 painting to induce Th2-type skin inflammation. We quantified numbers of OVA(257-264)/Kb$^+$ T cells within the skin of *CD8*^{cre} *Il4ra*$^{fl/-}$ and littermate control mice and determined

that Th2-inflammation impairs endogenous $CD8^+$ T_{RM} formation in an IL-4Rα-dependent, T cell-intrinsic manner (Extended Data Fig. 7d-g; page 13 line 332-342).

Again, we have revised Figure 4d to show the ratio of WT : *Il4ra*$^{-/-}$ $CD8^+$ $CD44^{hi}$ OT-I T cells recovered from each tissue rather than the ratio of skin : spleen in the hope that this presentation will be stronger. *Il4ra*-deficiency resulted in approximately two-fold the number of $CD8^+$ $CD44^{hi}$ OT-I T cells recovered from the spleen, with little difference between the number of *Il4ra*$^{-/-}$- and WT- OT-I T cells in bLN on day 8. However, *Il4ra*-deficiency resulted in approximately five-fold the number of OT-I T cells recovered from the skin. By day 30, while there were 1-6-fold more *Il4ra*$^{-/-}$ OT-I T cells than WT T cells in the spleen, there was a 7-55-fold increase in *Il4ra*$^{-/-}$ $CD8^+$ $CD44^{hi}$ $CD103^+$ OT-I T cells recovered from the skin. Additionally, we have now added Extended Data Fig. 6 to show absolute numbers of transferred T cells recovered from each tissue. We thank the reviewer for suggesting this data presentation, as it highlights the increase in *Il4ra*$^{-/-}$ OT-I T cells compared to WT OT-I T cells in the skin particularly at day 15, with a concomitant decrease in the bLN. These differences in recovery from the skin vs. dLN could reflect either/both increased recruitment of *Il4ra*$^{-/-}$ OT-I T cells from the bLN into the skin, or decreased exit of these cells from the skin. We now highlight these possibilities in the revised text ((page 12 line 307-319).

When we initially attempted these experiments, we used CD90.1/CD90.2 congenic markers, but unfortunately, the adoptively transferred T cells did not persist long-term in recipient mice in our hands (even without injection of depleting antibody). Of note, multiple groups have relied on CD8 antibody to deplete circulating memory T cells in order to determine the protective function of $CD8^+$ T_{RM} (Hobbs et al., PMID: 31801067; Shin et al., PMID: 27827367). Moreover, we have now performed plaque assays on skin tissue recovered from undepleted mice, and the results were not significantly different compared to mice injected with anti-CD8 antibody (Fig. 4g; page 14 line 360-365).

We have now included a naïve control group as well as undepleted mice (Fig. 4g; page 14 line 358-359). The high and low datapoints from the previous submission are not from separate experiments; there is variability within each experiment. We have included additional repeats of the experiment along with the additional controls that were requested, and while the data are variable within the experiments, the pooled data remain statistically significant.

Fig. 5. As stated above, since IL-4 can induce naïve T cell proliferation, is the decrease in TGFbRII expression that is observed in context of TGFb availability dependent on the number of cell divisions (if the cells were CFSE or CTV labeled, does the decrease occur after 1, 2, 3, etc. cell divisions and is it transient or sustained?) If the authors can make a stronger case that IL-4 signals reduce the Trm numbers in the skin and to the point that protection is affected, this could be a very exciting study. The paper would also benefit from some additional insight regarding the TGFbRII downregulation (see my comments re.Fig. 5 – when does it occur, how long does it last).

In these experiments, we activate the T cells for 4.5 days *before* culturing them with IL-4 with or without TGF-β. To determine whether their decrease in TGF-βRII expression is dependent on the number of cell divisions, we labeled the cells with CTV immediately before culturing them with IL-7 +/- IL-4 +/- TGF-β. After one day of culture, $CD8^+$ T cells cultured with either TGF-β alone or with TGF-β and IL-4 had both downregulated TGF-βRII expression (Extended Data Fig. 8, right panel; page 14 line 373-375), and they proliferated comparably based on CTV dilution (Extended Data Fig. 8, left panel; page 14 line 373-375).

Based on the reviewer's insightful suggestion, we also analyzed whether the TGF-βRII expression is transient or sustained. We observed that the gMFI of TGF-βRII expression decreased for $CD8^+$ T cells treated with either TGF-β alone or with TGF-β and IL-4. However, while the gMFI of TGF-βRII expression was restored on $CD8^+$ T cells treated with TGF-β at day 3, the gMFI remained lower on $CD8^+$ T cells treated with TGF-β and IL-4 (Fig. 5c; page 14 line 373-377).

Reviewer #2:

Remarks to the Author:

Mora-Buch and co-workers describe an inhibitory effect of IL-4 on TGFb-mediated induction of CD103 expression in activated CD8+ T cells that they link to impaired generation of CD103+resident memory CD8+ T cells in skin. While previous studies have focussed on tissue environmental factors that promote the generation of such cells, very little is known about factors that counteract their generation and maintenance. As such, their study is topical and of great interest. As outlined below however, there are several areas of concern that significantly dampen my enthusiasm for the presented work.

Major comments:

** Fig 2a: TGFb-mediated CD103 induction in WT cells in this graph is considerably less efficient compared to what is shown in Fig.1b/c/d and 3b (all with small error bars). In fact, Il4ra-/- andStat6-/- cells in Fig.2a show the same level of CD103 induction as the WT cells in Fig.1. Does this reflect different culture conditions between the experiments? Could the authors please clarify?*

The cells were cultured under the same conditions in these experiments. While we are not positive why there is a difference between the TGF-β-mediated CD103 induction in WT cells between experiments, one possible variable is different batches of TGF-β between experiments. Nonetheless, we include the WT $CD8^+$ T cells treated with TGF-β as a control for comparison to experimental conditions in every experiment. Although the magnitude of TGF-β-mediated CD103 induction in WT cells varies between experiments, the effects of *Il4ra*-defiency, *Stat-6*-deficiency, or treatment with TGF-β + IL-4 when compared to WT cells treated with TGF-β is consistent between experiments.

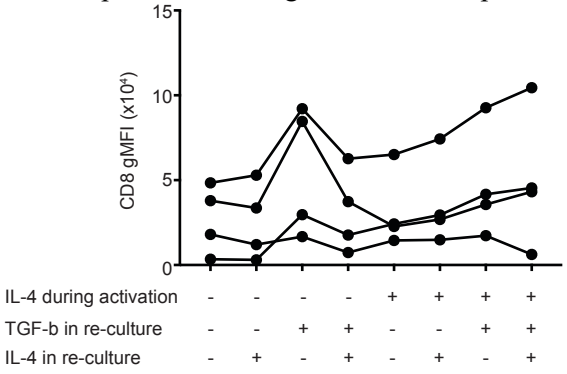
** Fig 2d: As the plots are pre-gated on CD8+ T cells, it would be a lot more informative to show CD44 versus CD103 expression, particularly since gating on CD44hi versus low can be a little arbitrary. This is also important given that IL-4 can impact thymic development of CD8+ T cells (work from Hogquist lab and others) and it is the thymus-egressing naïve CD44low cells that express intermediate levels of CD103.*

We have replotted the graphs to show CD44 vs. CD103 expression in the new Fig. 2e for $CD8^+$ T cells recovered from peripheral lymphoid tissues as well as from the thymus (page 7 line 188-193).

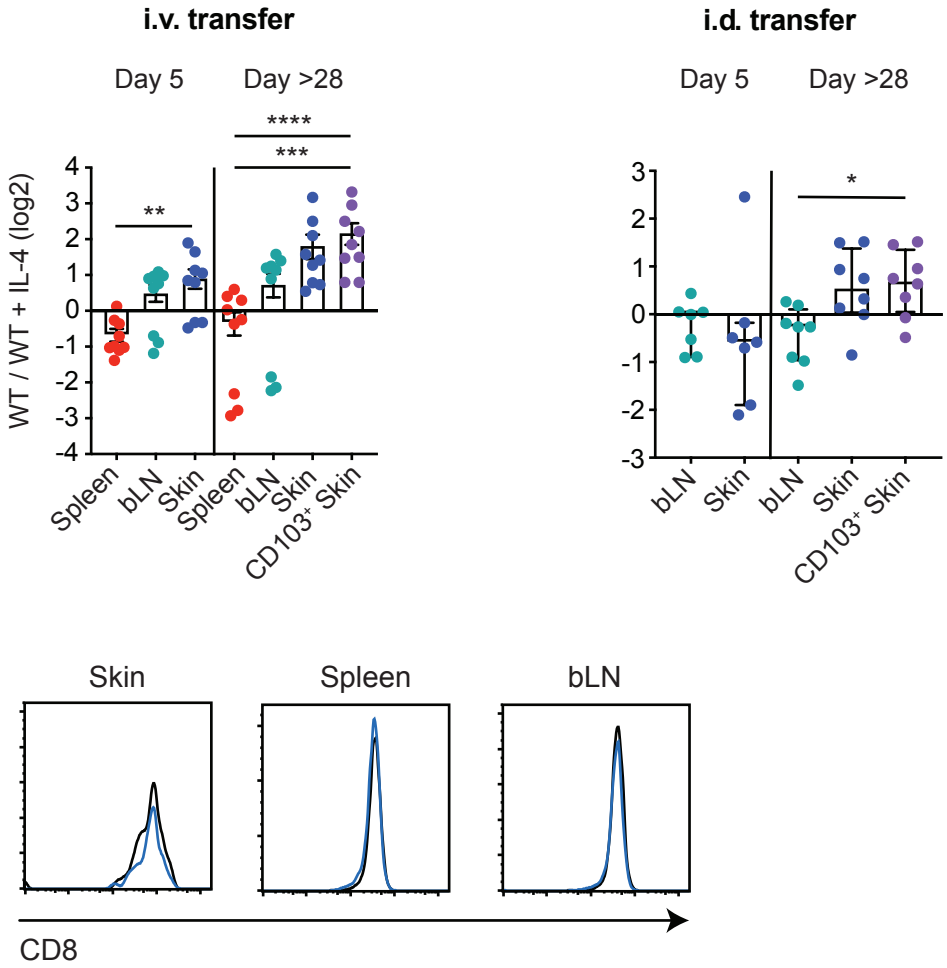
** Addition of IL-4 to activated CD8+ T cells can lead to profound and long-lasting downregulationof CD8 surface expression (see e.g. Erard F et al. Science 1993; Kienzle N et al. J Immunol 2005 ;and other several papers). Somewhat surprisingly, this is not evident from the FACS plots in Fig.1Band Fig. 3B, but I am assuming these are pre-gated on CD8+ events anyway? Regardless, can the authors rule out that this phenomenon, that is loss or reduction of CD8 expression after IL-4 stimulation, impacted on their enumeration of CD8+ T cells from tissues – particularly if results are shown as CD8-pregated events (also Fig. 3J/L and elsewhere)?*

First, we analyzed the gMFI of CD8 expression by $CD8^+$ T cells in the experiment depicted in Figure 3b. Briefly, $CD8^+$ T cells were initially activated by stimulation with anti-CD3 and anti-CD8 antibodies in the presence or absence of IL-4, and fed with fresh culture media and IL-2 on day 2 and 3. After 4.5 days, the $CD8^+$ T cells were isolated from the initial cultures and re-cultured with IL-7 +/- IL-4

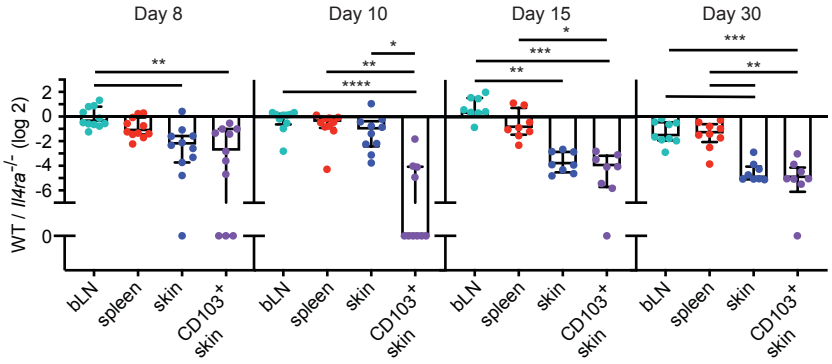
+/- TGF-b. Finally, after 3 days, the $CD8^+$ T cells were analyzed by flow cytometry. We did not detect a profound decrease in the gMFI of CD8 expression by $CD8^+$ T cells activated in the presence of IL-4 compared to $CD8^+$ T cells activated in its absence. We did observe that $CD8^+$ T cells activated in the absence of IL-4 and then re-cultured in the presence of IL-7 and TGF-β had an increased gMFI of CD8 expression compared to $CD8^+$ T cells re-cultured with IL-7 and IL-4 or with IL-7 and TGF-β + IL-4 in some experiments. The gMFI of CD8 expression from 4 experiments is shown below:



We have also re-analyzed Fig. 3 where $CD8^+$ T cells were activated in the presence or absence of IL-4 and then adoptively transferred into congenic recipient mice. In the data below, we gated on $CD3^+$ $CD44^+$ T cells, and then analyzed the fractions of $CD45.1^+$ and $CD45.2^+$ cells recovered from each tissue (without any CD8 pre-gating). We still observe an increased recovery of untreated compared to IL-4-treated $CD8^+$ T cells recovered from the skin after both i.v. transfer as well as i.d. transfer (top graphs). The flow cytometry plots depict CD8 expression by untreated (black line) and IL-4-treated (blue line) cells recovered from each tissue.



Finally, we also reanalyzed experiments in Fig. 4D by gating on $CD3^+$ $CD44^+$ T cells and then gating directly on $CD45.1^+$ and $CD45.2^+$ T cells, without pre-gating on $CD8^+$ cells. Again, we obtained similar results shown below.



** For experiments in Fig.4, could the authors please provide the rationale as to why they chose Il4ra-/- hosts instead of WT mice? The fact that IL4RA is not only missing in the transferred CD8 T cells but also in other immune cells (that may assist TRM generation) appears to unnecessarily complicate the interpretation of the results. Have they done similar experiments in WT mice?*

Please see our answer to reviewer #1's question 6 above.

** Fig. 4f: While the authors conclude that the modestly improved protection in Il4ra-/- mice is explained by a reduction in TRM numbers, an alternative or additional explanation could be that IL-4 inhibits or fine tunes antiviral CD8 effector functions. This is well documented in the literature (e.g. Erard-F et al, Science 1993; Sharma-DP et al, J Virol 1996; and many others) and complicates conclusions drawn from this experiment. Have they tested effector functions such as killing or cytokine production in these conditions? Also, what is the level of protection relative to non-transferred mice given that the OVAp tattoo will also generate endogenous OVA-specific TRM cells that should mediate protection?*

The reviewer makes an excellent point. We have examined effector functions, including cytokine production (IFN-γ, TNF-α) as well as CD107a expression in these conditions. These results are provided in Fig. 4e (page 13 line 343-353). We observed increased effector function by *Il4ra*$^{-/-}$ OT-I T cells compared to WT OT-I T cells in the spleen, but we did not detect significant difference in the skin. We predicted that the increase in effector function by circulating *Il4ra*$^{-/-}$ OT-I T cells would enhance clearance of replicating virus in mice whose CD8^{+} T cells were not depleted, and although some mice showed increased clearance of replicating HSV, we did not detect a significant difference (Fig. 4g; page 14 line 360-367).

We have also included a control of non-transferred mice (page 14 line 358-359). These mice are not protected as well as mice that also receive adoptive transfer of *Il4ra*$^{-/-}$ OT-I T cells.

** Fig.5A: IL-4 promotes downregulation of TGFR only in conjunction with TGFb stimulation. That is interesting but unfortunately this phenomenon remains unexplored.*

Thank you for highlighting this important question. We have included an additional experiment demonstrating that the gMFI of TGF-βRII expression by CD8^{+} T cells cultured with TGF-β + IL-4 decreases and remains decreased over time. In contrast, the gMFI of TGF-βRII expression by CD8+ T cells cultured with TGF-β alone initially decreases but is restored by day 3 (Fig. 5c; page 14 line 373-381). Previous studies have found that TGF-β rapidly and transiently induces Smad7 expression (Nakao et al., PMID: 9335507). Smad7 targets TGF-βRI for degradation (Kavsak et al., PMID: 11163210), leading to reduced cell surface TGF-βR expression. We wondered whether there might be a difference in Smad7 expression by T cells cultured in the different cytokine conditions. We found that on day 3, cells cultured with TGF-β + IL-4 expressed significantly increased Smad7 compared to CD8^{+} T cells cultured with either TGF-β or IL-4 alone, providing a possible mechanistic explanation for the robust decrease in TGF-βRII expression that we observed in cells cultured with TGF-β + IL-4 (Fig. 5a-d; page 14 line 373-386).

** The authors repeatedly state that "expression of the transcription factor, Eomes is downregulatedby TGFb-signaling" and seem to build some of their mechanistic framework around thisassumption. However, their own data in Fig.5C/D as well as some of the literature they cite (Nath etal, PLoS One 2019) do not appear to support this. On another note, the effects on Eomes areunrelated to TGFR modulation as IL-4 alone induces Eomes upregulation but not TGFRdownregulation. Overall, it remains largely unclear mechanistically as to how IL-4 may interfere withTGFb-mediated induction of CD103 in vitro or TRM cells in vivo.*

IL-4 can induce Eomes expression in $CD8^+$ T cells (PMID 27298446 and Figure 5f, g; page 15 line 393-394). Based on our *in vitro* data, this induction of Eomes expression by IL-4 is unrelated to TGF-βR modulation since we did not observe a significant change in TGF-βRII expression following culture of $CD8^+$ T cells with IL-4 alone (Figure 5a, b).

We thank the reviewer for pointing out that in contrast with published literature (PMID 35942952), we did not detect a significant decrease in the gMFI of Eomes expression following *in vitro* culture of $CD8^+$ T cells with TGF-β alone (Fig. 5f, g), despite a modest decrease in TGF-βRII expression by these cells (Fig. 5a, b). We have revised the text accordingly (page 15 line 391-392). On the other hand, when $CD8^+$ T cells were cultured with TGF-β and IL-4, TGF-βRII was significantly decreased compared to when cells were cultured with TGF-β alone. This more dramatic decrease in TGF-bRII expression correlated with a significant increase in Eomes expression compared to when cells were cultured with either TGF-β or IL-4 alone. Although we did not detect a decrease in Eomes expression following culture with TGF-β alone, we detected a significant increase in Eomes expression following culture with TGF-β and IL-4 (page 15 line 393-395).

Studies by Mackay et. al. (PMID: 26682984) used retrovirally transduced cells to overexpress Eomes, and these transduced $CD8^+$ T cells expressed decreased CD103 and formed fewer T_{RM} *in vivo*. Perhaps the significantly increased Eomes expression we observe following $CD8^+$ T cell culture with TGF-β and IL-4 parallels this Eomes overexpression. Indeed, we now include additional data that examines the gMFI of Eomes and CD103 expression in cutaneous WT- and *Il4ra*$^{-/-}$ $CD8^+$ OT-I T cells at a site of Th2 inflammation (Fig. 5h-j; page 15 line 395-401). Here, we measured Eomes and CD103 expression by adoptively co-transferred, *in vivo*-activated WT- and *Il4ra*$^{-/-}$- OT-I T cells recovered from MC903-induced atopic dermatitis lesions. 14 days post cell transfer, few WT OT-I T cells expressed CD103 (Fig. 5h), but the gMFI of Eomes expression in those WT OT-I cells that did express CD103 was similar to *Il4ra*$^{-/-}$ OT-I T cells (Fig. 5i, j). Of note, however, many WT OT-I T cells maintained high levels of Eomes expression and failed to express CD103 in this Th2-type microenvironment, consistent with a role for IL-4Rα signaling in preventing both Eomes downregulation and CD103 expression. In contrast, many *Il4ra*$^{-/-}$ OT-I T cells expressed CD103 but lacked Eomes expression. Moreover, among $CD103^-$ *Il4ra*$^{-/-}$ OT-I T cells, at day 14, many cells expressed intermediate Eomes, reminiscent of $CD8^+$ T cells transitioning to a $CD103^+$ phenotype (Mackay et al., PMID: 26682984) (Figure 5i). We have added discussion that our data suggest that IL-4Rα signaling leads to failure of $CD8^+$ T cells to downregulate Eomes expression *in vivo*, and this may prevent $CD8^+$ T_{RM} formation (page 15 line 395-401).

Our *in vitro* data also suggest that exposure of $CD8^+$ T cells to TGF-β and IL-4 results in prolonged downregulation of TGF-βR expression (Fig. 5c) and decreases pSmad2/3 phosphorylation (Fig. 5e). Because TGF-βR signaling is required for CD103 expression by $CD8^+$ T cells, this finding provides a mechanistic explanation for IL-4 interference with TGF-β-induced CD103 expression. Because both TGF-βR signaling and CD103 expression are required for $CD8^+$ T_{RM} formation, these findings provide an additional mechanism by which IL-4 may impair $CD8^+$ T_{RM} formation *in vivo* (page 14-15 line 370-386).

Minor comments:

** In many panels the authors show normalised data and sometimes even ratios thereof which makes it rather difficult to gauge the extent and robustness of the experimental differences. For instance, what does "normalised to recovery" mean (Fig.3/4)? Percentages? Numbers? Or"normalised to TGF condition" – for which genotype/s? Where feasible and applicable, the authors should also provide more easily interpretable values, e.g. % CD103+ cells, cell numbers etc.*

We have revised figures to include easily interpretable values, or in the case of normalized data (Fig. 1A, page 32 line 845-846; Fig. 1E, page 33 line 860-861; Extended Data Fig. 1A, supp page 1 line 4-5; Extended Data Fig. 1C, supp page 1 line 14-16), we have given the complete equation in the figure legend used to determine relative expression.

* Fig. 2c: Because the symbols overlay the bar graphs, I could not figure out the extent of the differences between the conditions. Can the authors please provide numbers as well, or enlargethe graphs?

We have replaced figure 2 with a new experiment using conditional *Il4ra*-deficient mice (new Fig. 2).

* Fig 2c: Is there a reduction in total CD8+ T cells as well, or is this specific for CD103-expressingCD8+ T cells?

We have replaced figure 2 with a new experiment using conditional *Il4ra*-deficient mice (new Fig. 2). We have made the symbols smaller in all graphs so that the bars can be seen more easily.

We would like to thank the reviewers for their complimentary comments on our revised manuscript. We have added the requested data in Extended Data Figure 9 and discussion with text in blue. Additionally, we have revised the text to shorten the manuscript while attempting to maintain clarity. Changes are annotated. We hope that our manuscript is now suitable for publication.

Reviewer #1

Just one last, small comment - in re to the Eomes expression observed here vs in previous studies. There is no consistency in the field when it comes to assessing the MFI of a population. Some groups compare the whole cell population (incl. cells that do not express the protein of interest), while other groups only examine MFI expression between the protein-expressing cells (gate on the + cells, then assess MFI). Also, some groups use the mean, some the geometric mean while others use the median fluorescence. It'd be good if the authors could make sure that the difference in Eomes expression (refs 19, 20) is not simply due to differences in MFI assessment.

We have added Extended Figure 9 to illustrate the mean and median fluorescence intensities of EOMES expression by $CD3^+$ $CD8^+$ T cells, as well as the mean fluorescence intensity of EOMES expression by $EOMES^+$ $CD3^+$ $CD8^+$ T cells. While we continue to observe significant differences in EOMES expression by $CD8^+$ T cells cultured with TGF-β versus with TGF-β and IL-4, we do not see a significant decrease in EOMES expression by $CD8^+$ T cells cultured with TGF-β versus with IL-7. We have also added qPCR analysis of CD8+ T cells cultured with IL-7 +/- TGF-β +/- IL-4.

It is possible that the differences in EOMES expression between our study and previously published studies result from differences in the experimental set-ups. While we cultured d4.5 *in vitro*-activated $CD8^+$ T cells with IL-7 and TGF-β, reference 15 (old reference 19) cultured d2 *in vitro*-activated $CD8^+$ T cells with IL-2 and TGF-β. IL-2 induces EOMES expression (reference 59), and so it is possible that the basal EOMES expression in these two culture systems is different. In the case of reference 16 (old reference 20), the investigators found that *Tgfbrii*$^{-/-}$ $CD8^+$ T cells isolated from the skin 14 days post-HSV infection expressed increased EOMES compared to wild-type $CD8^+$ T cells, suggesting that TGF-βRII receptor signaling decreases EOMES expression. Perhaps our *in vitro*-cultured $CD8^+$ T cell cultures do not replicate this *in vivo* infection model. We have discussed these possibilities in the discussion section ((page 16, lines 430-436).

Reviewer #2

Minor comment:
The title of Fig.2 appears to be wrong as it states: Fig. 2. IL-4 decreases CD103 expression and cutaneous CD8+ TRM numbers during homeostasis. There is no data in this Figure/study to support this statement.

Thank you for pointing out this error. We have revised the title of Fig. 2.