Peer Review File

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Manuscript Title: Metabolic Programs of T Cell Tissue Residency Empower Tumor Immunity

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

This manuscript by Reina-Campos offers an interesting new insight into the metabolic demands of a tissue-resident memory T cell in the intestine. The study finds through a crispr screen of metabolic enzymes a more selective dependency on cholesterol metabolism in SI Trm cells. The data are compelling that there is a heavy reliance on the mevalonate pathway in SI Trm cells and they authors try to uncover what that is. The main conclusion is that while it is not the synthesis of cholesterol per se that is important, it is the side reactions that lead to ubiquinone synthesis for example that is more important. They also try to manipulate cholesterol synthesis pathway selectively through inhibition of squalene synthetase fdft1 as a therapeutic to augment T cell responses in colon cancer models.

Overall view and major strengths and weaknesses: In general there is a lot of novelty in this study and it helps to highlight tissue-dependent and tissue-specific metabolic states for tissue resident immune cells, an area for which we know very little. I believe this work will be of high impact to the field and will serve as an foundational study to study other metabolic adaptations of tissue-resident immune cells, and one from which other new studies in the field will extend.

Some of parts of the manuscript are strongly supported by the data and other parts need more investigation, particularly as it relates to the hypothesis that the SI Trm cells are more reliant on ubiquinone synthesis. This is a very interesting idea, but there is very little data to support that. In fact, I think the manuscript in general would be stronger if it focused more on the infection-induced and dietary impacts of cholesterol on the Trm cells and less on the cancer models because that data is far less compelling. Why do the SI Trm cells die from higher cholesterol in the diet…does this lead to suppression of hpd, pdss1/2 and ubiquinone synthesis?

Also, it would be interesting for the authors to elucidate how Trm cells import cholesterol from the diet and if they have access to the lymphatics where the cholesterol would presumably go first as part of chylomicron packaging. In general, there is a lot of different directions packaged into this manuscript and it is easy for the reader to get lost, with some reorganization, I think the authors could streamline the main narrative and findings of the paper.

More detailed summary and suggestions: The authors use Srepbf2-KD and KO to investigate the effects of interfering with cholesterol synthesis on Trm development and find that while Trm in all tissues like liver and kidney are affected by Srebpf2 deletion, the Trm cells are the most affected by KD, and thus the interpretation is that the Trm cells in the intestine have the greatest dependence

on highest levels Srepbf2 activity. When they look at dietary import of cholesterol, the SI Trm import more, but exceeding normal levels in the diet reduces their numbers. WHY?? This is very interesting and could be of great relevance to obesity/statin use. What do statins do to Trm formation?

The low filipin staining in SI Trm is very interesting. Can the authors look at that with SREBP2 staining and how this is altered in the High and low cholesterol diets. Is there a difference in filipin staining in the LP vs SI Trm in the intestine?

They go onto explore further downstream aspects of the mevalonate pathway and focus on an enzyme fdft1 that synthesizes squalene one of the downstream steps of cholesterol synthesis because loss of fdft1 paradoxically increases Trm cell numbers in the liver, but not the SI. In contrast, loss of hpd and pdss1/2 have a defect in SI Trm formation. From this the authors conclude that other uses of mevalonate intermediates (such as ubiqunone synthesis) are critical for SI Trm cells development. This aspect of the paper is not further explored very much. Also, a confusing aspect about this interpretation is that there is still a strong demand for cholesterol synthesis in SI Trm because many genes downstream of fdft1 such as Sqle, msmo1, Dhcr24, Cyp51, Tm7sf2 are important for SI and liver Trm formation. They do an additional screen on Fdft1 KO cells and find other “arms” of the mevalonate pathway needed fro the increased survival seen by fdft1 KO. Here I felt like the study was going off on a hard to follow tangent because the differences in Fig. 3b appear small despite being statistically significant. Also, there is no further exploration of the ubiquinone pathway, but it would be strong to tie it to regulation of mitochondria function in Trm cells, especially if SI Trm cells are more reliant on particular types of ubiquinones.

Minor comments:

1) There are no labels on the extended data figs making it very difficult to review.

2) diagram of all the enzymes in the pathway with colors to show fold change in expression should be part of a Figure to help guide the reader.

3) What are the numbers of Trm cells in the high and low cholesterol diets. The numbers are presented relative to each other as opposed to control diet.

4) The analysis of liver Trm should not be filtered on iv-neg cells as liver Trm cells dwell in the sinusoids and therefore are labeled by the iv staining technique. Gating on CD69+ CXCR6+ cells in the liver is sufficient to capture the majority of the long-term liver-dwelling Trm cells.

Referee #2 (Remarks to the Author):

A and B: The manuscript by Reina Campos et al. describes metabolomic and transcriptomic adaptation of T-cells during their transition to tissue resident Trm cells. Of particular interest is the role of cholesterol biosynthesis (CB) and especially squalene synthase and its possible role in T-cell function. Overall, this manuscript adds to a recent body of literature highlighting the importance of distal cholesterol biosynthesis and its regulation for immune cell function. Conceptually this report might also partially explain anti-tumor activities of several inhibitors of distal CB and raises questions/opportunities for novel therapeutic concepts as primed here with ZAA. In general this is a very thoroughly conducted study.

C and D: methods appear sound as does statistical analysis.

Nevertheless, I have a couple of questions that I would like to raise, the will concern E and F

E and F:
Major
Generally speaking, the authors identify SREBP2 but entirely focus their investigation on polar metabolites. It is well known that SREBPs are important regulators of fatty acid and cholesterol biosynthesis. In turn, I wonder why did the authors not also conduct a lipidomics profiling of these cells. Ext F1d does actually proof my point as several fatty acids are even (judged by eye) more consistently upregulated, e.g. arachidonate, palmitate. Particularly arachidonate would also funnel into Ptges a gene identified in F1b. Along these lines and I understand that this is not always easy/possible but the choice for specific targets for subsequent evaluation does not always become clear to me. I would like to encourage the authors trying to make this even more clear to the reader. Another example would be several enzymes from distal cholesterol biosynthesis, e.g. EBP, CYP51 and DHCR24 are significantly downregulated in SITrm under shSREBf2 treatment but are not mentioned in the text. This is also true for the fact that both SI Trm and kidney Trm both have a strong correlation with mevalonate/chol biosynthesis in Ext F1a but to a lesser extend when looking at Ext F1f. Could the authors please explain/clarify. Moreover, the accumulation of fatty acids as shown in ext F1 speaks against a sole dependency on SREBP2 can this be explained by RUNX3? If specifically SREBP2 is activated how can FA accumulation be explained? Along these lines I wonder are all genes/enzymes of the entire Kandutsch Russel/Bloch pathway under control of SREBP2? If so why were they not regulated in extF5 – feeding experiments? One last experimental remark would be a better integration of transcriptomics with proteomics (metabolomics). To this end the study relies heavily on transcriptomics data without giving evidence for the actual regulation of the most important target proteins on the protein level. It might be worthwhile giving basic western blot evidence in SI Trm for the main proteins identified, GGPS, Squalene synthase and 4-hydroxyphenylpyruvate dioxygenase.

Hpd pathway, it is not that I don't agree with the authors line of argumentation here but coming back to my comment above, many enzymes (genes) are correlated wit SI Trm in F3. The authors argue that 4-HB is found in the metabolomics data and this is further explored. However, we again see many enzymes of distal CB and I wonder if the intermediates of this pathway can even be monitored with the applied metabolomics analysis. Generally speaking such closely related neutral lipids are hard to discover using untargeted metabolomics screens. My basic (general) question is, could it not also be that an entire class of metabolites is being neglected here while we know that

intermediates and products of distal CB are important effectors of T-cells via LXR? I understand that the CRISPR experiments in F3 target this question however it seemed important to me raising it. This also comes from the fact that unsaturated fatty acids are important precursors for many signalling lipids and as F1b identified Ptges and given the fact that LXR is known to affect PUFA composition I was wondering if there is not another possible link here. Particularly Ptges increase in SI Trm seems noteworthy as it funnels into recent reports on PGDH inhibitors as IBD therapy, but I understand this is a related but different topic.

Minor
Abstract
The authors state “pharmacological targeting” please specify inhibition vs. activation for me both targets the enzyme but in an opposing fashion.
Identification of 4-HB – have the retention times been matched against a genuine standard? The MS/MS data of the HB isomers will likely be identical so this MS matching figure is not really conclusive.

G: references are fine.
H: See comments above, maybe a "summary figure" would be nice for the reader and enhance clarity.

Referee #3 (Remarks to the Author):

This is a nice study of the metabolic profile of Trm focusing on the small bowel and then applying the findings to cancer immunity. The authors use a selected CRISPR screen to pull out drivers of Trm formation using a cell-autonomous model, which opens the door to the functional role of specific enzymes and intermediates along the mevalonate pathway and enzymes driving this. They then address whether this can affect the control of transplanted tumours in mice using additionally a drug blockade. They draw conclusions about the significance of this pathway in establishing Trm populations and how it can be potentially modulated in vivo.

I thought it was clearly laid out in general for condensing such a lot of data and a complex area, and they resolve some apparent inconsistencies (although there are a fair few typos, some of which impacted on the sense). The advance seems important as on the one hand we still do not have a complete grip on fate choices for Trm and on the other there looks like translational potential.

I had 3 areas that felt to me incomplete and/or confusing:

1. Linking the Trm to the tumor immunology: The thrust of the main part of the paper is on SI Trm and these appear to be the most distinctive. But the tumor work is in entirely different no GI tumour

types using a subcutaneous transplant system. The two bits did not seem to link instantly together. Some data on skin Trm would be a good intermediary and some more clearly available data on the phenotypes of cells in the tumors and the role of the pathways in individual cell types (ie local Trm vs other populations) would be helpful. There are some data on human and mouse transcripts by scRNASeq in the supplementary data but as displayed they are really difficult to evaluate.

2. Defining the cellular effect: The authors show an effect of the pathways in the screen, but it wasn't clear what the actual mechanistic role of the metabolites/pathways were in cellular function – that seems important in understanding the protective role and the impact on the tumours. Is the key functional effect related to proliferative capacity and just impacts on the cell number or other anti-tumour/host defence functions? The very condensed dataset in supp fig 7d suggest some of this is linked to strong proliferation (ki67 expression). The actual function of tumour specific cells (Trm and others) in the context of fdft1 CRISPR or ZAA therapy could be explained better.

3. Clarifying/Expanding the human data: There are some data on humans dotted about but it is hard to make sense of and it does seem important to get clear. The histology data (extended Fig 2e) are hard to interpret as there are not standard Trm markers in there. To what extent is the association CD8 specific and Trm specific? Similarly with the Extended Fig 7d gene expression data discussed above, this is lacking context. Could the authors clarify the data that suggest these pathways are active in human Trm cells in tissues and cancers - and that the same drug/gene targeting could be impactful. There is some start on this in Extended Fig 7a, but the figure legend doesn't really explain what is being examined and actually there doesn't seem to be much enrichment of the relevant pathway. That analysis needs to be performed on the Trm vs non-Trm subsets in the gut – there are some quite good published datasets to re-evaluate.

Referee #4 (Remarks to the Author):

In the manuscript by Reina-Campos and colleagues, the authors perform an innovative screen on genes in several metabolic pathways, to identify pathways that are important for the development/maintenance of tissue resident memory CD8 T cells (TRM). Based on the enrichment of genes in the Mevalonate and Cholesterol Synthesis pathways in small intestine (SI) TRM, the focus on the role of this pathway in SI TRM. In Fig 1, the authors focus down their screen to the Mevalonate and Cholesterol Synthesis pathway and the gene Srebp2, which is a key enzyme in the pathway. It is unclear if the original screen identified Srebp2. In figure 2, the authors perform several competitive knockdown and knockout experiments to highlight the role of Srebp2 and an upstream enzyme Scap in SI TRM, although the exact mechanism for the loss of Srebp2 KD/KO cells is not addressed in this figure. The authors correlate the uptake of dietary, but not circulating, cholesterol with the SI TRM, suggesting this is the pathway for their uptake of cholesterol. In Figure 3, the authors identify Fdft1 as a KO with the opposite phenotype of Srebp2 and Scap, and perform a

clever epistatic screen on Fdft1, to identify geranylgeranylation, tRNA modifications, and as potential downstream pathways. The authors then focus on the role of Srebp2 in T cells in subcutaneously transplanted tumors (MC38 and B16), showing an impressive impact of Fdft1 KO in MC38 and a modest effect of an inhibitor (ZAA) and PD-1 blockade on B16 tumors. These data seem out of place as the premise of the manuscript is that Srebp2 and the Mevalonate and Cholesterol Synthesis pathways are critical for TRM in the SI, but the tumor experiments are in the skin, where no data are shown otherwise. Moreover, the authors note the importance of this pathway and Srebp2 in T cell expansion, which is evidenced by the decreased expansion of T cells in spleen at day 7 in both LCMV infection (2b) and at day 5-7 in LM infection (2c). Thus, it is not clear that the tumor effects are at all connected to the TRM effects, as these could be distinct phenomena where Srebp2 is important for effector T cell expansion of T cells in tumors and for the generation/maintenance/survival of SI TRM. In line with this, the mechanism for the decreased TRM in SI in Srebp2 KO/KD T cells could be completely unrelated to the finding that Fdft1 KO has better tumor control in the skin. Moreover, much of the mechanism remains unclear in the study, as it remains unclear where the defects are impacting the SI TRM. This is somewhat addressed by the data in 2a, but 2b/2c argue for a broader impact of Srebp2 on all T cells, regardless of compartment. This discrepancy leaves open the possibility that Srebp2 has multiple roles in the biology of T cells, impacting T cell expansion, effector functon(?), tissue residence, etc. Some clarity is necessary for the paper to have a cohesive message. However, the largest conceptual issue with the manuscript is that figure 4 seems unrelated to figures 1-3. The authors are clearly trying to demonstrate that the deficiency in SI TRM is meaningful, but would be better served by testing a challenge that SI TRM are involved in directly, or focusing on the role of Srebp2 and the pathway described in skin TRM, with a challenge into skin that already contained wt or Srebp2 KO TRM. Without these more direct connections, the paper seems to be lacking in a cohesive message and is probably not ready for publication.

Additional points:

1. Line 136-137, figure 2a:
Authors showed the change of P14 number in different tissues, but it's not shown whether these P14 are tissue memory T cells. They claimed that "Resulted in impaired TRM formation in the SI, but not kidney, WAT, or liver", even though these P14 are TRM. Why? The reduced TRM may be caused by impaired formation of TRM, or reduced formation of effector T cells (since the source of Trm is not clear). Thus, the authors should use more careful time course analysis to better enumerate the numbers of each type of population (effector vs. TRM) in the tissues at different timepoints. Transfer experiments of equal numbers of WT and Srebp2 KO effector CD8 T cells with subsequent analysis of TRM formation would be the most direct means to address the question.

2. Line249-250:
The relation of SI Trm's acclimation to niche and the requirement of non-steroidal products like ubiquinone is not shown. Perhaps this is known in the field, but the mechanism should be verified by altering the level of non-steroidal metabolites (i.e., provide the T cells with additional ubiquinones, or OE ubiquinones synthesis enzymes to rescue the defect in Trm formation).

3. Figure 4,
The authors showed increased representation of Ftfd1 KO P14 in tumor and better tumor control,

but the mechanism remains unclear. The authors do not show functional analyses on the differences in TIL between wt and Ftfd1 KO and determine why the tumors are smaller. More to the point, with ZAA (Ftfd1 inhibitor), this could act on the T cells or tumor cells or other cells in the tumor. Moreover, ZAA will increase ubiquinone accumulation, and there is a concern regarding which TIL subpopulation is impacted. Is this really a TRM population or expansion of effector T cells? Is there even a ZAA-sensitive population in the tumor and is this a TRM? An alternative explanation is that because ubiquinone is important for the mitochondrial electronic transport chain, and exhausted T cells have mitochondrial dysfunction, it is possible that the ZAA inhibitor is impacting the process of exhaustion, not altering TRM. Likewise, it could impact effector function and increase the potency of effector T cells in tumors. Or ZAA could enhance migration into the tumor tissue (from the margin), or into the tissue in general. Or ZAA could increase proliferation of T cells in the tumor, which would allow them to better compete with the highly proliferative tumor cells reduce tumor growth. On their own, these would be interesting phenomena, but they would not be directly related to the data in Figs 1-3. The authors need to show whether these are distinct or similar phenomena and determine how the ZAA impacts the T cells vs. tumor cells.

4. The title of the work is confusing, as it suggests metabolic regulation is important for tissue memory T cell formation/maintenance/migration and that this is relevant for TIL. However, the case best made for SI TRM and cholesterol. It is not certain that there are TRM in the skin that rely on this pathway or that the phenomenon in tumors is related to the phenomenon in the SI TRM. The best data shown is that Ftdt1 inhibition/ko leads to smaller tumors, but how this is achieved is not clear. Specifically, why would the formation/maintenance/ migration of TRM into tumor be related to smaller tumors (as opposed to increased effector function), and if the mechanism is increased effector function, is that the same mechanisms maintaining/forming/migrating TRM into the SI? Seems likely to be at least two distinct phenomena, and that this metabolic pathway is important for many aspects of T cell biology.

Author Rebuttals to Initial Comments:

Common Metabolic Adaptations Empower CD8 T Cell Tissue Residency and Antitumor Immunity
Reina-Campos et al.
Point by Point Response to Referees

We sincerely thank the 4 reviewers for their time and effort in reading our manuscript and providing constructive and helpful criticism. We appreciate the positive feedback received from all four referees, stating our study is of "high impact", "foundational for the field", with "lots of novelty", "that offers an interesting new insight into the metabolic demands of a tissue-resident memory T cell in the intestine", “very thoroughly conducted", "clearly laid out in general for condensing such a lot of data and a complex area", and highlighted the high translational potential", the " innovative screen on genes in several metabolic pathways", and our "clever epistatic screen".

We have addressed all the referees' comments and have modified our manuscript and figures accordingly. We now provide a new figure showing the cellular effect of manipulating the mevalonate/cholesterol synthesis pathway and the underlying mechanism related to ubiquinone production (**new Fig. 4**). In addition, we provide new data reinforcing that T_{RM} and TIL have a common requirement for increased non-steroidal products of the mevalonate/cholesterol synthesis pathway and share an underlying mechanism controlling *in vivo* persistence related to producing non-steroidal metabolites, including ubiquinone (**new Fig. 5**). We have generated extensive new evidence showing that enforcing ubiquinone synthesis through Pdss2 overexpression is sufficient to increase mitochondrial respiration (**new Fig. 4**), amplify effector and memory CD8 T cell formation in the context of infection (**new Fig. 2**), and enhance the intratumoral accumulation and tumor control against a poorly immunogenic and aggressive tumor model (B16 melanoma) (**new Fig. 5**). Also, we have added new data showing that mice lacking T cell expression of Scap show impaired protection from LCMV rechallenge in the small intestine compared to WT mice (**new Fig. 2e**). We have streamlined our analysis of human T_{RM} and TIL datasets to show that the mevalonate/cholesterol synthesis pathway is more active in human T_{RM} than in circulatory or memory populations (**new Extended Data Fig. 2d-f**) and in human TIL, and T_{RM}-like TIL than circulatory and memory populations (**new Extended Data Fig. 7d**). We have also refined our presentation of mouse TIL populations (**new Extended Data 7**) and have added a new analysis that supports our expanded discussion regarding the dissociation of the activation of the mevalonate/cholesterol synthesis pathway with cellular proliferation in T_{RM} and highly functional TIL in response to reviewer suggestions (**new Extended Data Fig. 8**). Finally, we have included new data regarding the effect of pharmacologically targeting the mevalonate/cholesterol synthesis pathway through statins in mice and humans (**new Fig. 2**). We believe our study has improved substantially as a result. Please, find our point-by-point response below:

Referee #1:

This manuscript by Reina-Campos offers an interesting new insight into the metabolic demands of a tissue-resident memory T cell in the intestine. The study finds through a crispr screen of metabolic enzymes a more selective dependency on cholesterol metabolism in SI Trm cells. The data are compelling that there is a heavy reliance on the mevalonate pathway in SI Trm cells and they authors try to uncover what that is. The main conclusion is that while it is not the synthesis of cholesterol per se that is important, it is the side reactions that lead to ubiquinone synthesis for example that is more important. They also try to manipulate cholesterol synthesis pathway selectively through inhibition of squalene synthetase fdft1 as a therapeutic to augment T cell responses in colon cancer models.

Overall view and major strengths and weaknesses: In general there is a lot of novelty in this study and it helps to highlight tissue-dependent and tissue-specific metabolic states for tissue resident immune cells, an area for which we know very little. I believe this work will be of high impact to the field and will serve as an foundational study to study other metabolic adaptations of tissue-resident immune cells, and one from which other new studies in the field will extend.

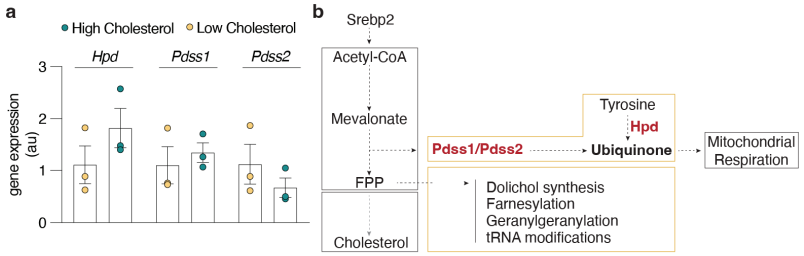
Some of parts of the manuscript are strongly supported by the data and other parts need more investigation, particularly as it relates to the hypothesis that the SI Trm cells are more reliant on ubiquinone synthesis. This is

a very interesting idea, but there is very little data to support that. In fact, I think the manuscript in general would be stronger if it focused more on the infection-induced and dietary impacts of cholesterol on the Trm cells and less on the cancer models because that data is far less compelling.

1) Why do the SI Trm cells die from higher cholesterol in the diet…does this lead to suppression of hpd, pdss1/2 and ubiquinone synthesis?

To answer the second question first; high cholesterol diet (7 days of treatment) does not impact the expression of *Pdss1*, *Pdss2*, nor *Hpd* in SI IEL CD8 T cells (**Referee Figure 1a**). Also, according to our transcriptional analysis of Srebf2-deficient P14 CD8 T cells in the context of infection, (**Extended Data Fig. 4)**, the loss of Srebf2 does not significantly change the expression of *Hpd*, *Pdss1*, or *Pdss2*. These data suggest that *Hpd*, *Pdss1*, and *Pdss2* are not directly controlled by Srebp2 activity, and thus their expression does not depend on sensing cholesterol levels.

Regarding the mechanism behind the toxicity of high cholesterol diet on SI CD8 T cells; Under our proposed working model (**Referee Figure 1b**), high cholesterol would result in a reduction in Srebp2 activity, due to the well-established negative feedback mechanisms of this pathway. Thus, these CD8 T cells would have reduced production of intermediates of the mevalonate/cholesterol pathway that feed into the production of non-steroidal metabolites, such as ubiquinone, produced by Hpd, Pdss1, and Pdss2, impairing the capacity of SI T_{RM} to acclimate to the tissue, and resulting in lower T_{RM} formation.



Referee Figure 1. **a**, qPCR of indicated genes in CD8 T cells isolated form the SI of mice fed a low cholesterol or a high cholesterol diet for 7 days. **b**, Schematics of the regulation model of the mevalonate/cholesterol synthesis pathway by Srebp2 that feeds into the production of non-steroidal metabolites, such as ubiquinone. Data are mean +/- s.e.m. (**a**).

2) It would be interesting for the authors to elucidate how Trm cells import cholesterol from the diet and if they have access to the lymphatics where the cholesterol would presumably go first as part of chylomicron packaging.

We thank the referee for their suggestion. Indeed, lipid import mechanisms of T_{RM} are an important and promising area of research that we are actively pursuing, but beyond the scope of this study.

In general, there is a lot of different directions packaged into this manuscript and it is easy for the reader to get lost, with some reorganization, I think the authors could streamline the main narrative and findings of the paper.

We have worked hard to focus the manuscript on key points and generally make the ideas more accessible.

More detailed summary and suggestions: The authors use Srepbf2-KD and KO to investigate the effects of interfering with cholesterol synthesis on Trm development and find that while Trm in all tissues like liver and kidney are affected by Srebpf2 deletion, the Trm cells are the most affected by KD, and thus the interpretation is that the Trm cells in the intestine have the greatest dependence on highest levels Srepbf2 activity.

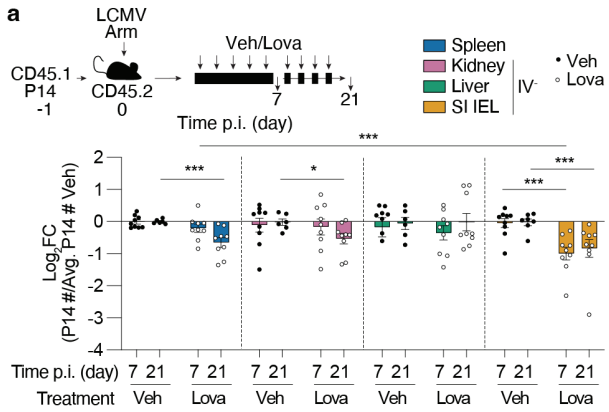
3) When they look at dietary import of cholesterol, the SI Trm import more, but exceeding normal levels in the diet reduces their numbers. WHY?? This is very interesting and could be of great relevance to obesity/statin use.

We thank the reviewer for sharing their enthusiasm about our study. Regarding the mechanism behind the toxicity of high dietary cholesterol, under our proposed model, high cholesterol would result in a reduction in Srebp2 activity, which would lower the generation of pathway intermediates that feed into the production of non-steroidal metabolites, such as ubiquinone, impairing the capacity of SI T_{RM} to acclimate to the tissue, and resulting in lower T_{RM} formation. We agree that this is an unexplored and important aspect of our study, and we have added new data regarding statin use in new **Fig. 2f,g**.

4) What do statins do to Trm formation?

We thank the author for this very insightful comment. We have added new data to Figure 2 (new **Fig. 2f, and Referee Figure 2a**) showing that Lovastatin treatment in mice prevents SI and Kidney T_{RM} formation after LCMV infection. In addition, we have added new data to Figure 2 showing that SI CD8 T cells from humans on statin

medication have lower T_{RM} scores than non-statin users (new **Fig. 2g and Referee Figure 2b**). These data strongly reinforce that mevalonate/cholesterol synthesis is important for T_{RM} formation in the intestine, and of high potential significance in humans.



Referee Figure 2. **a**, Quantification of total P14 CD8 T cells from indicated tissues at day 7 and 21 after LCMV infection in mice treated with vehicle or Lovastatin (Lova) as indicated. Log_2FC scores were calculated by comparing P14 numbers in each sample to the average number of P14 in Veh-treated mice. **b**, T_{RM} scores of human CD8 T cells of the spleen and SI profiled by scRNAseq grouped by statin use of their respective human donors. Data are mean +/- s.e.m. (**a**) and geometric distribution (**b**), and representative of at least two independent experiments (**a**) with a total of n=9. Un-paired t-Test (**a**,**b**). **P*<0.05, ***P*<0.01, ****P*< 0.005

The low filipin staining in SI Trm is very interesting:

6) Can the authors look at that with SREBP2 staining and how this is altered in the High and low cholesterol diets?

We thank the referee for their suggestion. Unfortunately, we could not achieve Srebp2 staining to capture translocation to the nucleus by imaging flow.

7) Is there a difference in filipin staining in the LP vs SI Trm in the intestine?

[redacted]

[Redacted Figure]

8) They go onto explore further downstream aspects of the mevalonate pathway and focus on an enzyme fdft1 that synthesizes squalene one of the downstream steps of cholesterol synthesis because loss of fdft1 paradoxically increases Trm cell numbers in the liver, but not the SI. In contrast, loss of hpd and pdss1/2 have a defect in SI Trm formation. From this the authors conclude that other uses of mevalonate intermediates (such as ubiqunone synthesis) are critical for SI Trm cells development. This aspect of the paper is not further explored very much. Also, a confusing aspect about this interpretation is that there is still a strong demand for cholesterol synthesis in SI Trm because many genes downstream of fdft1 such as Sqle, msmo1, Dhcr24, Cyp51, Tm7sf2 are important for SI and liver Trm formation.

This is an excellent point that we have now addressed extensively. First, we have added new data showing that Pdss2 overexpression (OE) is sufficient to induce higher memory CD8 T cell accumulation after LCMV infection (new **Fig. 3e**), promote mitochondrial respiration (new **Fig. 4e**), ubiquinone production (new **Fig. 4g**), higher intratumoral accumulation in MC38-GP_{33-41} and enhanced tumor control of B16-GP_{33-41} melanoma tumors. These new data offer conclusive evidence that enhancing ubiquinone production is a common approach to enhancing CD8 T cell persistence (**Referee Figure 4**).

To gain a better insight into the common metabolic adaptations of SI T_{RM} and TIL, and clarify the requirement for cholesterol production, we then performed a secondary targeted screen utilizing our 50 gene mevalonate/cholesterol synthesis pathway library. The new data added to Figure 5 (new **Fig. 5a-c**) shows that

SI T_{RM} and TIL (but not CD8 T cells in the draining Lymph Node, dLN) share a similar reliance on enzymes related to the production of non-steroidal metabolites, including those involved in ubiquinone synthesis. Of note, Dhcr7 and Dhcr24, the last two enzymes that synthesize the final cholesterol molecule in the Bloch and the Kandutsch & Russell pathways, respectively, did not affect SI T_{RM} formation or TIL accumulation (new **Fig. 5c**). These data combined with our targeted screen, our initial screen, the dietary intervention, the transcriptional and Imaging Flow profiling, and nSrebp2 overexpression experiments, strongly suggest SI T_{RM} do not require intrinsic synthesis of cholesterol.

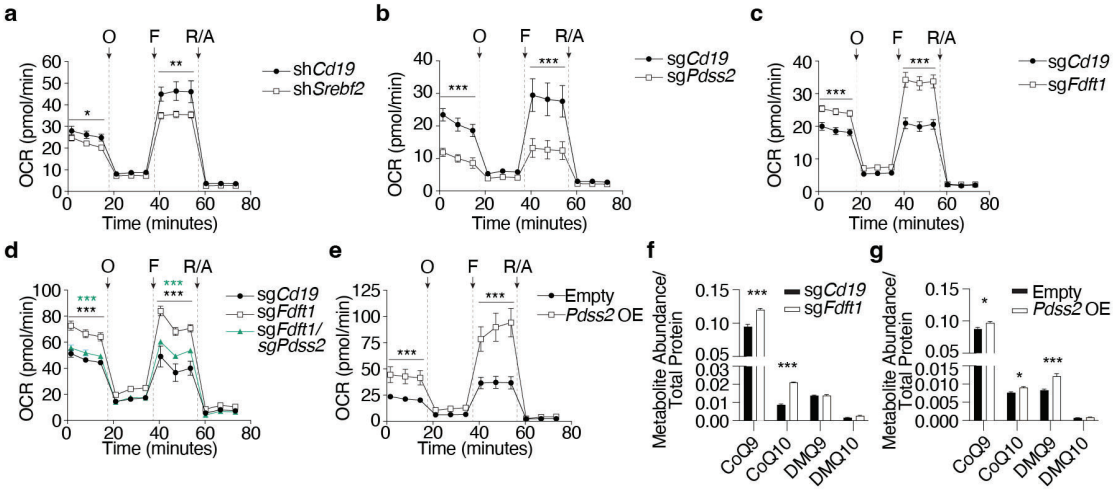
Thus, we have clearly shown that cholesterol synthesis is not required. Given the intricacies of this pathway and the strong feedback and feedforward regulatory loops that exist, it could be that the enzymes mentioned by the referee as apparent inconsistencies in our initial screen could exert an effect on SI T_{RM} by modulating upstream enzyme activities or may have additional functions beyond cholesterol synthesis. We believe unraveling this interesting complexity is important but beyond the scope of this study given the conclusive results regarding cholesterol synthesis. We have now further clarified these points in the manuscript.

9) They do an additional screen on Fdft1 KO cells and find other "arms" of the mevalonate pathway needed from the increased survival seen by fdft1 KO. Here I felt like the study was going off on a hard to follow tangent because the differences in Fig. 3b appear small despite being statistically significant.

We apologize for the lack of clarity and have reworked this section. Regarding Figure 3b, Hpd KO P14 CD8 T cells have a ~2-fold average reduction in their capacity to form SI T_{RM}. The effect observed is significant and specific for SI T_{RM}. This result has been further replicated as *Hpd* was the 7th most required enzyme for SI T_{RM} formation (Log2FC of -10) in our new targeted CRISPR/Cas9 screen (new **Fig. 5a-c**). The epistatic screen in Fdft1 KO cells revealed *Pdss2* as a critical step in cells with elevated ubiquinone production, which we believe offers useful insights into how this pathway is regulated in memory CD8 T cells in vivo.

10) Also, there is no further exploration of the ubiquinone pathway, but it would be strong to tie it to regulation of mitochondria function in Trm cells, especially if SI Trm cells are more reliant on particular types of ubiquinones.

We have added new data showing that lack of mevalonate/cholesterol synthesis pathway reduces the basal and maximal mitochondrial respiration capacity of CD8 T cells (new **Fig. 4a and Referee Fig. 4a**). Also, we have added new data showing that the increased mitochondrial function endowed by Fdft1 loss is dependent on Pdss2 (new **Fig. 4b-d and Referee Fig. 4b-d**). In addition, and as mentioned in response to point 8 above, Pdss2 OE is sufficient to increase the basal and maximal respiration capacity of the mitochondria of CD8 T cells concomitant to an increase in CoQ9, CoQ10, and the CoQ9 precursor DMQ9. Unfortunately, the reliance on



Referee Figure 4. **a,** Oxygen consumption rate (OCR) of vitro activated sh*Cd19* and sh*Srebf2* CD8 T cells subjected to the MitoStres test (Seahorse). **b**, OCR of in vitro-activated sg*Cd19* and sg*Pdss2* Cas9eGFP CD8 T cells subjected to the MitoStress test (Seahorse). **c**, OCR of in vitro activated sg*Cd19* and sgFdft1 Cas9eGFP CD8 T cells subjected to the MitoStress test (Seahorse). **d**, OCR of in vitro-activated sg*Cd19*, sg*Fdft1, and* sg*Fdft1/Pdss2* Cas9eGFP CD8 T cells subjected to the MitoStress test (Seahorse). **e**, OCR of in vitro activated empty vector control (Empty) and *Pdss2* OE CD8 T cells subjected to the MitoStress test (Seahorse). **f**, Quantification of ubiquinone and demethoxyubiquinone species in in vitro activated sg*Cd19* and sg*Fdft1* Cas9eGFP CD8 T cells normalized to total protein content. **g**, Quantification of ubiquinone and demethoxyubiquinone species in in vitro activated Empty and Pdss2 OE CD8 T cells normalized to total protein content. Data are mean +/- s.e.m and representative of at least two independent experiments (**a**-**g**). Two-way

particular types of ubiquinones could not be further explored, as we currently lack the technical approaches to deliver titrated amounts of different types of ubiquinone species to the right cellular compartments. Mostly due to the highly hydrophobic nature of ubiquinone, we could not find a valid strategy to provide exogenous ubiquinone during our Seahorse measurements.

Minor comments:

1) There are no labels on the extended data figs making it very difficult to review.

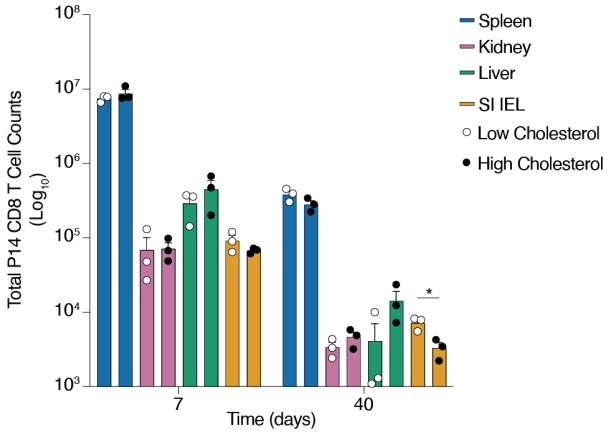
We apologize for this omission. We have now added labels to all figures.

2) Diagram of all the enzymes in the pathway with colors to show fold change in expression should be part of a Figure to help guide the reader.

We thank the referee for their suggestion. Our expanded additional schematic diagram in **Extended Data Fig. 6a** covers the pathways investigated in detail. After carefully considering different visualization strategies, we believe adding the fold change values for each enzyme for the 6 cellular populations included in the initial screen to the diagram does not improve clarity. As an effort to improve clarity, we have added an additional diagram in Figure 5 (new **Fig. 5d**) to aid in interpreting results from our new screen. Of note, we also provide a more simplified schematic in **Extended Data Fig. 6g**, and a summary scheme in new **Fig. 5m**.

3) What are the numbers of Trm cells in the high and low cholesterol diets. The numbers are presented relative to each other as opposed to control diet.

This particular experimental setup compared a low cholesterol diet and high cholesterol diet, which are isocaloric diets specifically designed for this experiment. The individual numbers of T_{RM} in their respective diets are illustrated below (**Referee Figure 5**):



Referee Figure 5. Total P14 CD8 T cell counts by time point after LCMV infection in mice subjected to a low cholesterol or a high cholesterol diet. Data are mean +/- s.e.m. All tissues, but the spleen, were gated on IV$^-$ P14 CD8 T cells. Un-paired T-test. *P<0.05

4) The analysis of liver Trm should not be filtered on iv-neg cells as liver Trm cells dwell in the sinusoids and therefore are labeled by the iv staining technique. Gating on CD69+ CXCR6+ cells in the liver is sufficient to capture the majority of the long-term liver-dwelling Trm cells.

We chose to focus on IV$^-$ P14 CD8 T cell populations (or IV$^-$ CD44^{high} Tetramer$^+$ CD8 T cells) to be able to gate on comparable populations across tissues, as previously done in similar publications from our laboratory and others (Crowl and Heeg et al., 2021 Nat. Imm., Anderson et al., 2014, Nat. Protocols, Skon et al., 2013 Nat. Imm.). We agree with the referee that IV stain does not fully capture the complexity of liver T_{RM}. We have included a mention in the methods to acknowledge the presence of IV$^+$ T_{RM} that might not be accounted for throughout our study, but we could not add these data for the studies that have been completed.

Referee #2 (Remarks to the Author):

A and B: The manuscript by Reina Campos et al. describes metabolomic and transcriptomic adaptation of T-cells during their transition to tissue resident Trm cells. Of particular interest is the role of cholesterol biosynthesis (CB) and especially squalene synthase and its possible role in T-cell function. Overall, this manuscript adds to a recent body of literature highlighting the importance of distal cholesterol biosynthesis and its regulation for immune cell function. Conceptually this report might also partially explain anti-tumor activities of several inhibitors of distal CB and raises questions/opportunities for novel therapeutic concepts as primed here with ZAA. In general this is a very thoroughly conducted study.

C and D: methods appear sound as does statistical analysis.

Nevertheless, I have a couple of questions that I would like to raise, the will concern E and F:
Major
Generally speaking, the authors identify SREBP2 but entirely focus their investigation on polar metabolites. It is well known that SREBPs are important regulators of fatty acid and cholesterol biosynthesis.

1) In turn, I wonder why did the authors not also conduct a lipidomics profiling of these cells. Ext F1d does actually proof my point as several fatty acids are even (judged by eye) more consistently upregulated, e.g. arachidonate, palmitate.

[Redacted]

[Redacted Figure]

2) Particularly arachidonate would also funnel into Ptges a gene identified in F1b. Along these lines and I understand that this is not always easy/possible but the choice for specific targets for subsequent evaluation does not always become clear to me. I would like to encourage the authors trying to make this even more clear to the reader.
We have modified the manuscript to make our rationale of focusing on major pathways, rather than individual genes, much clearer to the reader. As a note, we appreciate the interest of this reviewer in *Ptges*. We will make all these data publicly available and easily searchable at the Functional Immunometabolism and Transcriptomics Database (FITDb) at the time of publication so other researchers can follow up on specific targets of their choosing, choose the best sgRNAs, and better explore our data.

3) Another example would be several enzymes from distal cholesterol biosynthesis, e.g. EBP, CYP51 and DHCR24 are significantly downregulated in SITrm under shSREBf2 treatment but are not mentioned in the text.
We have added new data showing that Dhcr7 and Dhcr24, the last two enzymes that synthesize the final cholesterol molecule in the Bloch and the Kandutsch & Russell pathways, respectively, do not affect SI T_{RM} formation or TIL accumulation (**new Fig. 5c**). These data combined with our targeted screen, our initial screen, the dietary intervention, the transcriptional and Imaging Flow profiling, and nSrebp2 overexpression experiments,

strongly suggest SI T_{RM} do not require intrinsic synthesis of cholesterol. However, as direct targets of Srebp2, the downregulation of EBP, CYP51, and DHCR24 is expected in Srebf2 KD cells.

This is also true for the fact that both SI Trm and kidney Trm both have a strong correlation with mevalonate/chol biosynthesis in Ext F1a but to a lesser extend when looking at Ext F1f. Could the authors please explain/clarify.
We realize the confusion might arise from labeling IV^- P14 CD8 T cells from the Kidney at day 7 as "Kidney T_{RM}", when in fact, at this time point, these cells are mostly effector CD8 T cells. We have modified the label "Kidney T_{RM}" to "Kidney IV^- P14 CD8 T cells" at effector time points after LCMV infection. To further clarify, Kidney T_{RM} have much lower mevalonate/cholesterol synthesis pathway scores in two different published datasets (**Extended Data Fig. 1a and Fig. 1g**) and a third dataset generated in this study (**Extended Data Fig. 4b**). These data are in agreement with our metabolomics data. **Extended Data Fig. 1a** does show elevated mevalonate/cholesterol synthesis pathway scores for "Kidney T_{RM}" (now labeled IV^- P14 CD8 T cells) at day 7 after LCMV infection. This is consistent with the effector phenotype of these cells and the data generated in this study (**Extended Data Fig. 4b).**

4) Moreover, the accumulation of fatty acids as shown in ext F1 speaks against a sole dependency on SREBP2 can this be explained by RUNX3?
Our transcriptomics and functional CRISPR/Cas9 analysis showed no increased or particular reliance of T_{RM} on enzymes of fatty acid synthesis, including *Fasn*. In relation to point 1, our data showed SI T_{RM} have a higher amount of lipids. We have not explored the contribution of Runx3 to this phenotype as it is beyond the scope of this study.

5) If specifically SREBP2 is activated how can FA accumulation be explained?
[Redacted]

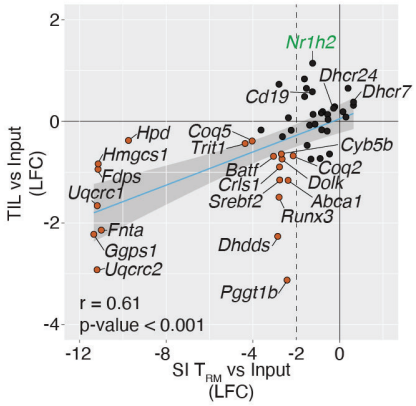
[Redacted Figure]

6) Along these lines I wonder are all genes/enzymes of the entire Kandutsch Russel/Bloch pathway under control of SREBP2? If so why were they not regulated in extF5 – feeding experiments?
For the feeding experiments, we chose three bona fide direct targets of Srebp2 to test whether Srebp2 activity is modulated upon increasing dietary-derived cholesterol. The expression of these genes was measured by qPCR on isolated CD8 T cells from the spleen and SI. This study was not designed to measure whether other enzymes of this pathway might be direct targets of Srebp2. Our **Extended Data Fig. 4c** shows the impact of Srebf2 deletion on the expression of all genes included in the mevalonate/cholesterol synthesis pathway, including Dhcr24, and Dhcr7.

7) One last experimental remark would be a better integration of transcriptomics with proteomics (metabolomics). To this end the study relies heavily on transcriptomics data without giving evidence for the actual regulation of the most important target proteins on the protein level. It might be worthwhile giving basic western blot evidence in SI Trm for the main proteins identified, GGPS, Squalene synthase and 4-hydroxyphenylpyruvate dioxygenase.
We thank the referee for their advice. Unfortunately, we are very limited by the number of viable T_{RM} isolated from mice tissues. We usually recover up to 10,000 P14 CD8 T cells from the SI of one mouse on day 21 after infection. For one western blot analysis of one SI T_{RM} sample, we would require using 100 mice, assuming 1 million CD8 T cells are needed for enough protein yield. Thus, total of 600 mice would be required for at least technical triplicates in two independent biological repeats. Unfortunately, we do not have access to fresh human tissue, and are very limited by the quality of commercial antibodies needed to detect these proteins. We would

like to note to this referee that we provide evidence of increased HMGCR protein expression in CD8 T cells of the SI and colon in humans compared to the spleen (**Extended Data Fig. 2f**), and have added new functional data to profile the metabolic requirements of SI T_{RM} and TIL (**new Fig. 5a**).

8) Hpd pathway, it is not that I don't agree with the authors line of argumentation here but coming back to my comment above, many enzymes (genes) are correlated with SI Trm in F3. The authors argue that 4-HB is found in the metabolomics data and this is further explored. However, we again see many enzymes of distal CB and I wonder if the intermediates of this pathway can even be monitored with the applied metabolomics analysis. Generally speaking such closely related neutral lipids are hard to discover using untargeted metabolomics screens. My basic (general) question is, could it not also be that an entire class of metabolites is being neglected here while we know that intermediates and products of distal CB are important effectors of T-cells via LXR?
We thank the reviewer for this very insightful comment and agree with the importance of clarifying this point. We agree that we are limited by our ability to annotate the metabolomics data. As mentioned also to referee 1 in point 8, to gain a better insight into the common metabolic adaptations of SI T_{RM} and TIL, clarify the requirement for cholesterol production, and additional downstream, and upstream regulators (such as LXRB), we have performed a secondary targeted screen utilizing our 50 gene mevalonate/cholesterol synthesis pathway library. The new data added to Figure 5 (**new Fig. 5a-c**) shows that SI T_{RM} and TIL (but not CD8 T cells in the draining Lymph Node, dLN) share a similar reliance on enzymes related to the production of non-steroidal metabolites, including those involved in ubiquinone synthesis. Of note, *Nr1h2* (LXRB), which is included in the targeted library, was found not to have a significant effect on the formation of SI T_{RM} or TIL, nor in our previous screen in Fdft1 KO CD8 T cells (**new Fig. 3d**, **new Fig. 5a**, and provided a highlight in **Referee Fig. 8**). As a remark, *Hpd* was again found as a top positive regulator of SI T_{RM} formation (L2FC of -10) (**Referee Fig. 5a-c**). In addition, we have performed lipidomic profiling to measure ubiquinone species on *Fdft1* KO and *Pdss2* OE CD8 T cells. Our new data (**new Fig. 4 and Referee Figure 4**) showed that both *Fdft1* deletion and *Pdss2* OE increase the production of CoQ9, CoQ10, and the ubiquinone precursor DMQ9 (new **Fig. 4f,g**).



Referee Figure 8. Log_2FC of sgRNA frequencies in outputs versus inputs for SI TRM formation (x axis) versus TIL accumulation (y axis). 19 significant genes in the overlap are colored dark orange, Nr1h2 highlighted in green.

9) I understand that the CRISPR experiments in F3 target this question however it seemed important to me raising it. This also comes from the fact that unsaturated fatty acids are important precursors for many signalling lipids and as F1b identified Ptges and given the fact that LXR is known to affect PUFA composition I was wondering if there is not another possible link here. Particularly Ptges increase in SI Trm seems noteworthy as it funnels into recent reports on PGDH inhibitors as IBD therapy, but I understand this is a related but different topic.
We agree with the referee and would hope to explore these possibilities in future experiments.

Minor
Abstract
1) The authors state "pharmacological targeting" please specify inhibition vs. activation for me both targets the enzyme but in an opposing fashion.
This omission has now been corrected in the text

2) Identification of 4-HB – have the retention times been matched against a genuine standard? The MS/MS data

of the HB isomers will likely be identical so this MS matching figure is not really conclusive.
Yes, a genuine standard was used with >97.5% clarity by HPLC (Millipore Sigma Ref #91554). We have now added a brief note on the methods.

G: references are fine.

3)
H: See comments above, maybe a "summary figure" would be nice for the reader and enhance clarity.
We thank the referee for this suggestion. An additional diagram and additional summary figures are now included in the **new Fig. 5**.

Referee #3 (Remarks to the Author):

This is a nice study of the metabolic profile of Trm focusing on the small bowel and then applying the findings to cancer immunity. The authors use a selected CRISPR screen to pull out drivers of Trm formation using a cell-autonomous model, which opens the door to the functional role of specific enzymes and intermediates along the mevalonate pathway and enzymes driving this. They then address whether this can affect the control of transplanted tumours in mice using additionally a drug blockade. They draw conclusions about the significance of this pathway in establishing Trm populations and how it can be potentially modulated in vivo.

I thought it was clearly laid out in general for condensing such a lot of data and a complex area, and they resolve some apparent inconsistencies (although there are a fair few typos, some of which impacted on the sense). The advance seems important as on the one hand we still do not have a complete grip on fate choices for Trm and on the other there looks like translational potential.

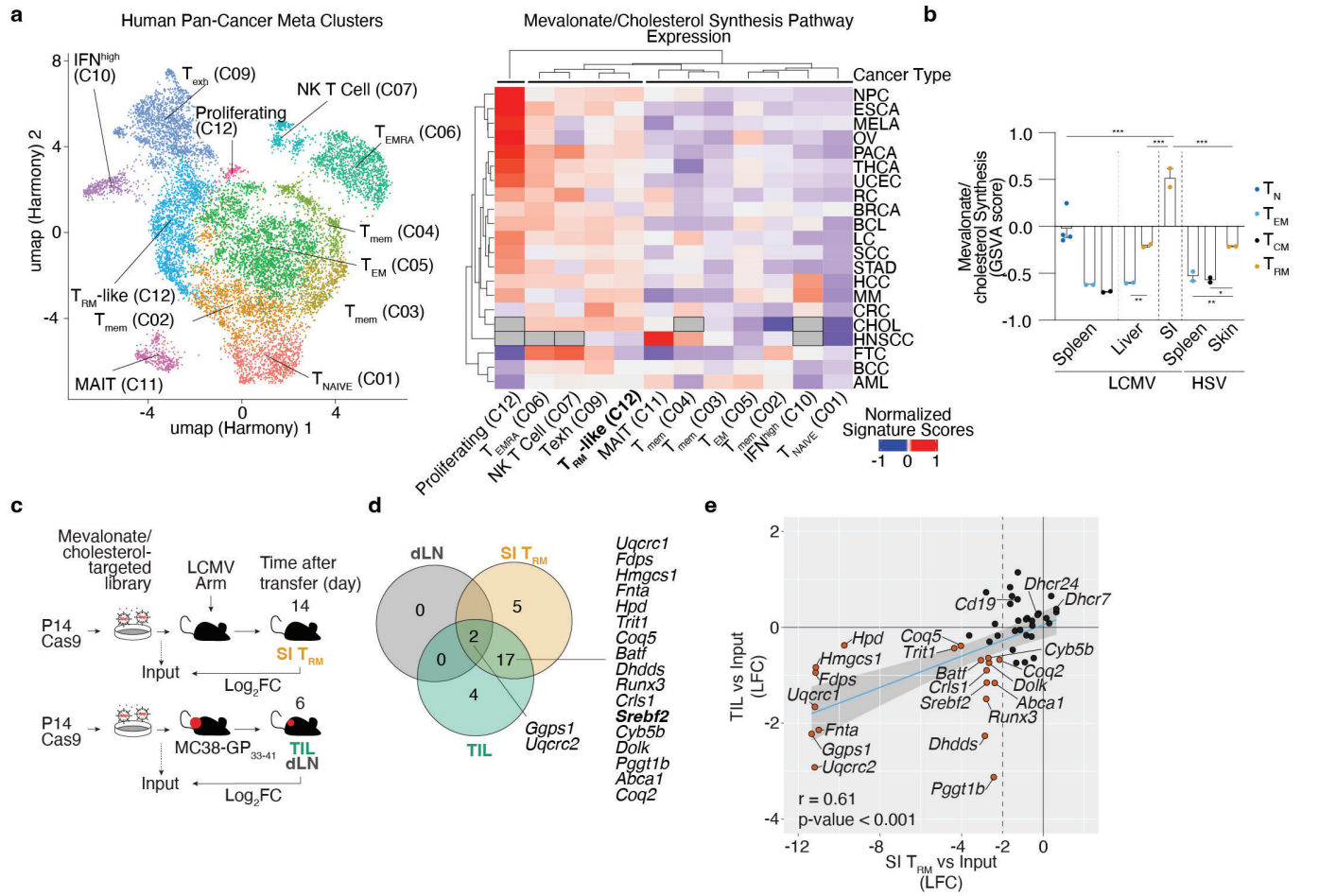
I had 3 areas that felt to me incomplete and/or confusing:

1. Linking the Trm to the tumor immunology: The thrust of the main part of the paper is on SI Trm and these appear to be the most distinctive. But the tumor work is in entirely different no GI tumour types using a subcutaneous transplant system. The two bits did not seem to link instantly together.

- *Some data on skin Trm would be a good intermediary and some more clearly available data on the phenotypes of cells in the tumors and the role of the pathways in individual cell types (ie local Trm vs other populations) would be helpful.*
- *There are some data on human and mouse transcripts by scRNASeq in the supplementary data but as displayed they are really difficult to evaluate.*

We appreciate the feedback on how to better convey our findings. We have removed the data from the CRC datasets and have now included new data using a recently published pan-cancer T cell atlas across 21 human cancer cell types (Zheng et al., Science 2021). These data show that the mevalonate/cholesterol synthesis pathway is almost universally increased in CD8 T cell populations mostly found intratumorally compared to circulating populations of CD8 T cells (**new Extended Data Fig. 7d and Referee Figure 9a**). In addition, these new data better delineate which TIL clusters have increased expression of the mevalonate/cholesterol pathway. We would like to note to this referee that data on mouse skin T_{RM} was included in **Extended Data Fig. 2c.** Skin T_{RM} have significantly higher expression of mevalonate/cholesterol pathway synthesis genes than circulatory memory cells (**Extended Data Fig. 2c and Referee Figure 9b)**. Thus, while the mevalonate/cholesterol synthesis pathway activity is highest in SI T_{RM}, the upregulation of this pathway in TIL does not appear to be tissue-specific. This is an important point that has been added to the manuscript. As a note, we would like to point out that our experimental tumor models, MC38-GP_{33-41} and B16-GP_{33-41} tumors, were implanted subcutaneously, not intradermally. Subcutaneous implantation of these tumor cell lines is a widely used and accepted approach to studying general tumor immunological responses, and does not necessarily reflect tissue immunity of the skin, while intradermal injection does (**Park et al., 2018, Nature**).

Finally, to gain a better insight into the common metabolic adaptations of SI T_{RM} and TIL, and to more solidly "link the T_{RM} to tumor immunology", we have performed a secondary targeted screen utilizing our 50 gene mevalonate/cholesterol synthesis pathway library. The new data added to Figure 5 (**new Fig. 5a-c and Referee Figure 9 c-e**) shows that SI T_{RM} and TIL (but not CD8 T cells in the draining Lymph Node, dLN) share a similar reliance on enzymes related to the production of non-steroidal metabolites, including those involved in ubiquinone synthesis. These data agree with previously published functional screens using similar libraries (**Extended Data Fig. 8a-c**).



mevalonate/cholesterol synthesis pathway scores grouped by "meta.cluster.coarse" labels and cell type. NPC, nasopharingeal carcinoma, ESCA, esophageal cancer, LC, lung cancer, HCC, hepatocellular carcinoma, CHOL, cholangiocarcinoma, RC, renal carcinoma, CRC, colorectal cancer, AML, acute myeloid leukemia, BCL, B-cell lymphoma, MM, multiple myeloma, HNSCC, head and neck squamous cell carcinoma, THCA, thyroid carcinoma, MELA, melanoma, BCC, basal cell carcinoma, SCC, squamous cell carcinoma, BRCA, breast cancer, STAD, stomach adenocarcinoma, PACA, pancreatic cancer, OV, ovarian cancer, FTC, fallopian tube carcinoma, UCEC, uterine corpus endometrial carcinoma. EMRA, effector memory CD45RA+, mem, memory. **b**, Mevalonate/cholesterol synthesis GSVA scores for indicated subsets of CD8 T cells at the indicated tissues in the context of LCMV and Herpes Simplex Virus (HSV), GSE70813. **c**, Experimental design of the in vivo CRISPR/Cas9-mediated loss-of-function screen of mevalonate/cholesterol pathway synthesis enzymes for SI TRM after LCMV infection, and dLN and TIL accumulation in MC38-GP_{33-41} tumors. **d**, Overlap of statistically significant positive regulators of SI TRM formation and CD8 T cell accumulation in dLN and tumors, related to a. **e**, Log_2FC of sgRNA frequencies in outputs versus inputs for SI TRM formation (x axis) versus TIL accumulation (y axis). 19 significant genes in the overlap are colored dark orange. Data are mean +/-

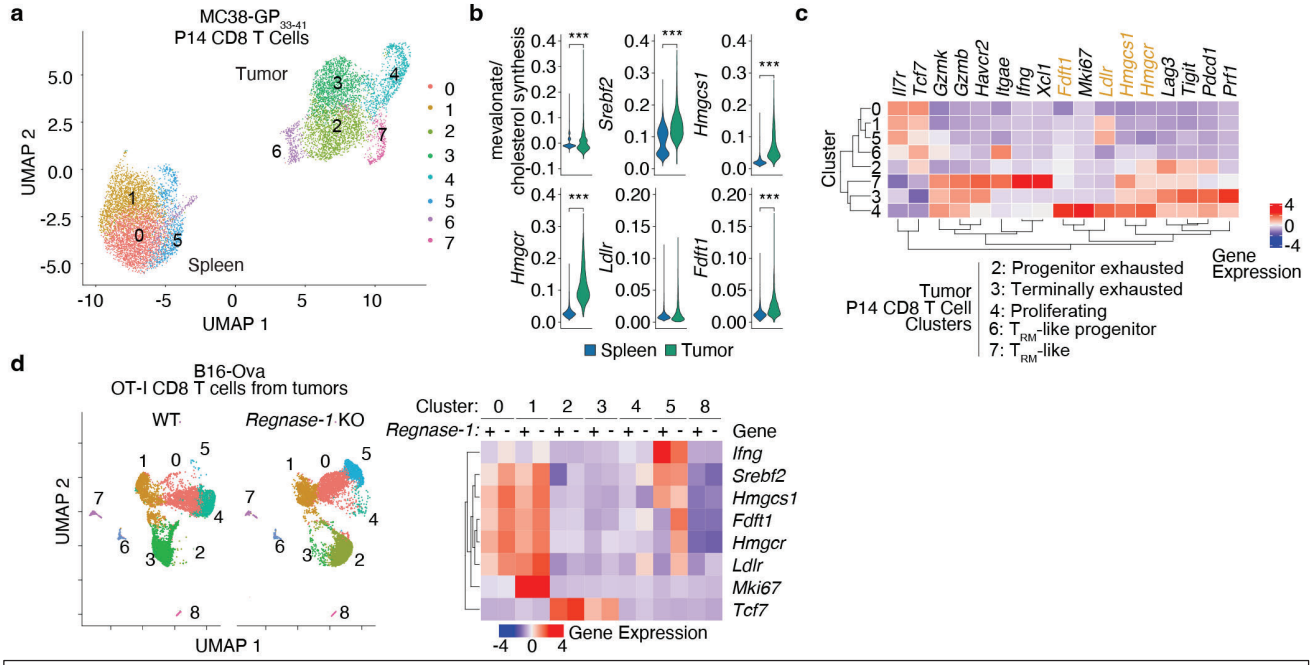
2. Defining the cellular effect: The authors show an effect of the pathways in the screen, but it wasn't clear what the actual mechanistic role of the metabolites/pathways were in cellular function – that seems important in understanding the protective role and the impact on the tumours.

- *Is the key functional effect related to proliferative capacity and just impacts on the cell number or other anti-tumour/host defence functions?*

Our data suggest that the proliferative capacity is not impaired in *Srebf2*-deficient CD8 T cells (KD), as seen by the lack of differential expression of genes related to proliferation in SI T_{RM} upon Srebf2 KD (**Extended Data Fig. 4c**), and the lack of an effect on the accumulation of P14 CD8 T cells deficient for Srebf2 in the spleen in the

context of tumor challenge (**new Fig. 5e**), or the lack of an effect of *Srebf2* sgRNA on P14 CD8 T cell accumulation in the dLN (**new Fig. 5a-c**). Our new data suggest that the main cellular effect of this pathway on CD8 T cells is through modulating mitochondrial respiration. This is supported by new data showing that lack of mevalonate/cholesterol synthesis pathway reduces the basal and maximal respiration capacity of the mitochondria of CD8 T cells (**new Fig. 4a and Referee Figure 4a**). Also, we have added new data showing that the increased mitochondrial function endowed by Fdft1 loss is dependent on Pdss2 (**new Fig. 4b-d and Referee Figure 4b-d**). In addition, and as mentioned in response to the point 8 of referee 1, Pdss2 OE is sufficient to increase the basal and maximal respiration capacity of the mitochondria of CD8 T cells concomitant to an increase in CoQ9, CoQ10, and the CoQ9 precursor DMQ9. An enhanced ability to sustain elevated mitochondrial respiration rates is a well-known metabolic trait associated with enhanced memory formation capacity and heightened antitumor responses.

The very condensed dataset in supp fig 7d suggest some of this is linked to strong proliferation (ki67 expression). We thank the reviewer for bringing this important aspect of our study into our consideration. In line with previous literature, our data does show a clear link between increased mevalonate/cholesterol synthesis pathway with increased cellular proliferation (**Extended Data Fig. 2a, 7c,d, Referee Figure 9a and 10c**), consistent with the need for anabolism. In addition, our data also shows that mevalonate/cholesterol synthesis pathway increase can be dissociated from proliferation in certain CD8 T cell populations. These include T_{RM}, especially SI T_{RM}, which have low homeostatic proliferation (Jarjour et al., PNAS 2022), and in a distinct, very active, and highly antitumorigenic TIL population in the absence of increased proliferation, such as T_{RM}-like TIL (**Extended Data Fig. 7c and Referee Figure 10c**), or CD8 T cells that lack Regnase 1 (Wei et al., 2019 Nature) (**Extended Data Fig. 8a-c and Referee Figure 10d**). We hope the changes made to the new **Extended Data Fig. 7 and 8** are able to convey these observations better. We have also now added this to the discussion.



scRNAseq 7 days after adoptive cell transfer. **b**, Mevalonate/cholesterol synthesis signature values and expression of indicated genes in P14 CD8 T cells from the spleen and MC38-GP_{33-41} tumors profiled by scRNAseq. MAGIC-imputed gene values are shown. **c**, Hierarchically clustered heatmap of scaled averaged gene expression values by cluster of P14 cells profiled from spleens and MC38-GP_{33-41} tumors by scRNAseq 7 days after adoptive transfer. **c**, Dimensionality reduction plot by UMAP of WT and Regnase-1 KO OT-I cells profiled from MC38-GP_{33-41} tumors by scRNAseq 7 days after adoptive transfer (left) and unsupervised hierarchical clustering heatmap of selected genes related to CD8 T cell cytotoxicity (*Ifng*) stemness (*Tcf7*), proliferation (*Mki67*), and the mevalonate/cholesterol synthesis pathway (*Srebf2*, *Hmgcs1*, *Fdft1*, *Hmgcr*, and *Ldlr*) grouped by cell type and cluster. Data are geometric distribution of gene expression values (**b**). Un-paired T-test (**b**). **P*<0.05, ***P*<0.01, ****P*< 0.005

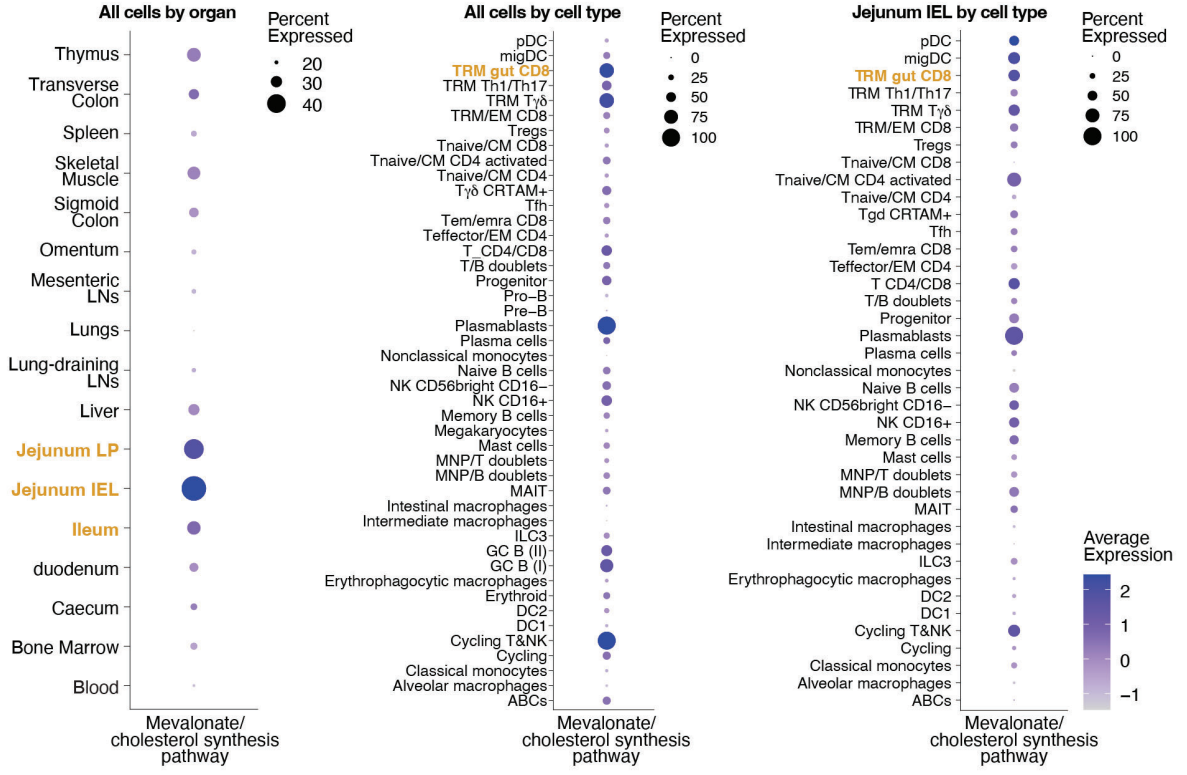
The actual function of tumour specific cells (Trm and others) in the context of fdft1 CRISPR or ZAA therapy could

be explained better.
We have modified the text accordingly

3. Clarifying/Expanding the human data: There are some data on humans dotted about but it is hard to make sense of and it does seem important to get clear. The histology data (extended Fig 2e) are hard to interpret as there are not standard Trm markers in there.

- *To what extent is the association CD8 specific and Trm specific?*

Given the limitations of histology to four channels, we could not add a fourth marker (CD103) to our current panel for histology (HMGCR, CD3, CD8a, DAPI). Given the reported phenotype of CD8 T cells in the human small intestine and colon (Szabo et al., 2017, Sci. Imm.), we believe our profiled CD8 T cells are composed mostly by T_{RM}. However, we agree with the reviewer that this is an important point to clarify. To this end, we reanalyzed a recently published cross-tissue immune dataset (Dominguez Conde, et al., 2022, Science). Our new data included in **Extended Data Fig. 2d** show that the increase in the mevalonate/cholesterol synthesis pathway is highest in immune cells of the small intestine, followed by other tissues, and minimal in immune cells from the spleen and the blood. By cell type, CD8 T_{RM} from the SI, plasmablasts, and proliferating T cells had the highest percentage and highest expression of enzymes of this pathway (**Extended Data Fig. 2d and Referee Figure 11**). To add granularity to the different immune cells present in the SI, we included a third comparison that showed CD8 T_{RM}, pDC, migratory DCs (migDC), γδT cells, plasmablasts, and proliferating T cells as the main populations with high expression of the components of this pathway (**Extended Data Fig. 2d and Referee Figure 11**).



Referee Figure 11. Mevalonate/cholesterol synthesis pathway scores for all human immune cells grouped by indicated tissue (left), cell types (middle), or cell types of the Jejunum SI (right), as profiled by scRNAseq in the tissue immune cell atlas.

- *Similarly with the Extended Fig 7d gene expression data discussed above, this is lacking context.*

We agree. We have removed these data and have added a more robust analysis encompassing 21 different cancer types. We believe the new data will convey our findings better.

- *Could the authors clarify the data that suggest these pathways are active in human Trm cells in tissues and cancers?*

The data supporting the mevalonate/cholesterol synthesis pathway being more active in human T_{RM} than in circulatory or memory populations are included in **new Extended Data Fig. 2d,e,f.** The data supporting the

mevalonate/cholesterol synthesis pathway being more active in human TIL, and T_{RM}-like TIL than circulatory and memory populations are included in **new Extended Data Fig. 7e**. We hope these newly added data and the restructuring of the former figures convey our findings better.

- *and that the same drug/gene targeting could be impactful. There is some start on this in Extended Fig 7a, but the figure legend doesn't really explain what is being examined and actually there doesn't seem to be much enrichment of the relevant pathway. That analysis needs to be performed on the Trm vs non-Trm subsets in the gut – there are some quite good published datasets to re-evaluate.*

We appreciate the referee's suggestions to improve the clarity of our results. We have restructured this section to simplify how data are presented from our scRNAseq of P14 CD8 T cells (**Referee Figure 10a-c**). To present our human data, instead of focusing on CRC, we have added new data from two recent pan-tissue (**Referee Figure 11**) and pan-cancer (**Referee Figure 9a**) scRNAseq atlases that offer a higher view of the activity of this pathway across tissues and cancer types, while preserving cell type granularity. As a note, CD8 T cells of the SI are virtually all tissue-resident as assessed by parabiosis experiments (**Steinert et al., 2015, Cell, Figure 3b**). In humans, most studies agree that the majority of CD8 T cells present in this tissue have a tissue-resident phenotype. Thus, an analysis of "Trm vs non-Trm subsets in the gut" cannot be performed. However, we hope this referee agrees that new data added to **new Extended Data Figure 2d,** together with the previous data in **Extended Data Figure 2e,f** make a good argument that SI T_{RM}, while not exclusively, have a distinct upregulation of the mevalonate/cholesterol synthesis pathway compared to CD8 T cells in the spleen and blood in humans.

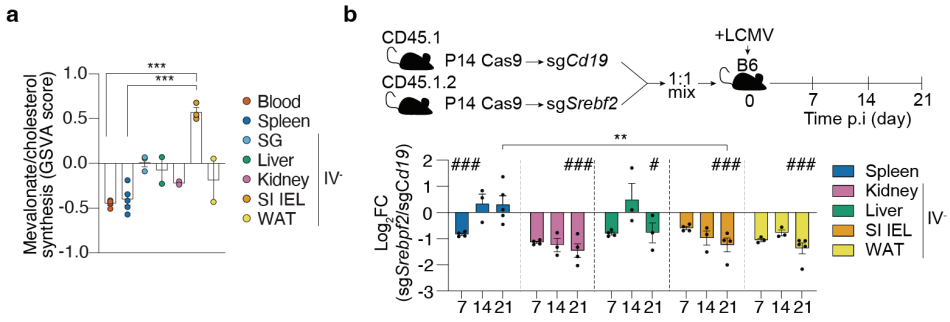
Referee #4 (Remarks to the Author):

In the manuscript by Reina-Campos and colleagues, the authors perform an innovative screen on genes in several metabolic pathways, to identify pathways that are important for the development/maintenance of tissue resident memory CD8 T cells (TRM). Based on the enrichment of genes in the Mevalonate and Cholesterol Synthesis pathways in small intestine (SI) TRM, the focus on the role of this pathway in SI TRM. In Fig 1, the authors focus down their screen to the Mevalonate and Cholesterol Synthesis pathway and the gene Srebp2, which is a key enzyme in the pathway. It is unclear if the original screen identified Srebp2. In figure 2, the authors perform several competitive knockdown and knockout experiments to highlight the role of Srebp2 and an upstream enzyme Scap in SI TRM, although the exact mechanism for the loss of Srebp2 KD/KO cells is not addressed in this figure. The authors correlate the uptake of dietary, but not circulating, cholesterol with the SI TRM, suggesting this is the pathway for their uptake of cholesterol. In Figure 3, the authors identify Fdft1 as a KO with the opposite phenotype of Srebp2 and Scap, and perform a clever epistatic screen on Fdft1, to identify geranylgeranylation, tRNA modifications, and as potential downstream pathways. The authors then focus on the role of Srebp2 in T cells in subcutaneously transplanted tumors (MC38 and B16), showing an impressive impact of Fdft1 KO in MC38 and a modest effect of an inhibitor (ZAA) and PD-1 blockade on B16 tumors.

1) These data seem out of place as the premise of the manuscript is that Srebp2 and the Mevalonate and Cholesterol Synthesis pathways are critical for TRM in the SI, but the tumor experiments are in the skin, where no data are shown otherwise. Moreover, the authors note the importance of this pathway and Srebp2 in T cell expansion, which is evidenced by the decreased expansion of T cells in spleen at day 7 in both LCMV infection (2b) and at day 5-7 in LM infection (2c).

We have worked hard to better present these findings, highlighting the idea that understanding T_{RM} metabolic programming informs strategies to enhance T cell persistance and targeting of tumors in general. Srebp2 and the mevalonate/cholesterol synthesis pathway are most upregulated in SI T_{RM}, but are also higher in Skin T_{RM} and Liver T_{RM} than their circulatory counterparts (**Fig. 1g and Referee Figure 12a**), and functionally relevant for Kidney, Liver, and WAT T_{RM} (**Fig. 2b and Referee Figure 12b**). Our new data show that expression profiles for relevant players of this pathway are similar across multiple human tissues (**Extended Data Fig. 2d and Referee Figure 11**). We have modified the text to make this point clearer. Regarding our experimental tumor model, MC38-GP_{33-41} and B16-GP_{33-41} tumors were implanted subcutaneously, not intradermally. Subcutaneous implantation of these tumor cell lines is a widely used and accepted approach to studying general tumor immunological responses, and does not necessarily reflect tissue immunity of the skin, while intradermal injection

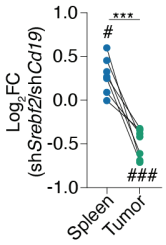
does (**Park et al., 2018, Nature**). Thus, our data show that adaptations that promote tissue residency also promote targeting of tumors, and enhancing the intermediates of the pathway, enhances T cell immunity to infection and tumors.



Referee Figure 12. **a**, Mevalonate/cholesterol synthesis pathway GSVA scores from RNAseq analysis of indicated P14 cells from indicated tissue >30 days after LCMV infection, GSE182276. SG, salivary gland. **b,** Congenically distinct P14 Cas9-eGFP+ were transduced with retroviruses containing an Srebf2 sgRNA (sgSrebf2) or a control (Cd19) sgRNA, mixed at a 1:1 ratio and transferred to recipient mice, subsequently infected with LCMV, and the ratio of transduced cells was evaluated by flow cytometry at 7, and 21 days in the indicated tissues. Data are mean +/- s.e.m. Un-paired t-Test (**a**,**b**).*P<0.05, **P<0.01, ***P< 0.005. one-sample t-Test (**b**). #P<0.05,

2) Thus, it is not clear that the tumor effects are at all connected to the TRM effects, as these could be distinct phenomena where Srebp2 is important for effector T cell expansion of T cells in tumors and for the generation/maintenance/survival of SI TRM.

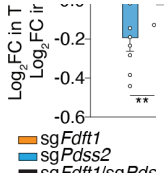
Our data suggest that the proliferative capacity is not impaired in Srebp2 deficient CD8 T cells (KD), as seen by the lack of differential expression of genes related to proliferation in SI T_{RM} upon Srebf2 KD (**Extended Data Fig. 4f**), and the lack of an effect on the accumulation of P14 CD8 T cells deficient for Srebf2 in the spleen in the context of tumor challenge (**new Fig. 5e)**. In addition, to clarify the connection between the effects of this pathway in SI T_{RM} and TIL accumulation, we have performed a secondary targeted screen utilizing our 50 gene mevalonate/cholesterol synthesis pathway library. The new data added to Figure 5 (**new Fig. 5a-c and Referee Figure 9c-e**) shows that SI T_{RM} and TIL (but not CD8 T cells in the draining Lymph Node, dLN) share a similar reliance on enzymes related to the production of non-steroidal metabolites, including those involved in ubiquinone synthesis. Of note, Srebf2 deletion did not significantly impact the accumulation of CD8 T cells in the dLN, suggesting CD8 T cell proliferation is no ıre 9c-e).



Referee Figure 13. Congenically distinct Cas9 P14 were transduced with retroviruses containing a *Srebf2* sgRNA (sg*Srebf2*) or a control (sg*Cd19*) sgRNA, mixed at a 1:1 ratio and transferred to MC38 GP_{33-41} tumor-bearing mice, and the ratio of transduced cells was evaluated by flow cytometry 7 days after adoptive transfer. Individual replicates shown. Un-paired t-Test. ***P< 0.005. one-sample t-Test. #P<0.05, ###P< 0.005

3) In line with this, the mechanism for the decreased T_{RM} in SI in Srebp2 KO/KD T cells could be completely unrelated to the finding that Fdft1 KO has better tumor control in the skin.

We now provide data showing that the increased accumulation of Fdft1 KO CD8 T cells in subcutaneously implanted tumors depends on Pdss2 expression (**new Fig. 5f and Referee Figure 14**), suggesting that a similar mechanism as the one found in liver T_{RM} in the context of infection applies to intratumoral CD8 T cell accumulation. These data are also corroborated by the fact that increased mitochondrial respiration upon deletion of Fdft1 depends on Pdss2 (**new Fig. 4b-d and Referee Figure 4b-d**).



Cas9 P14 CD8 T cells in MC38-GP_{33-41} tumors 7 days after adoptive cell transfer. Data are mean +/- s.e.m. Paired t-Test.

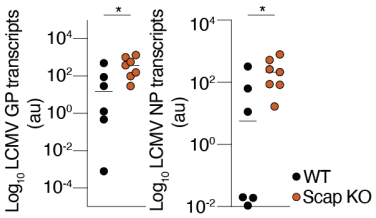
4) Moreover, much of the mechanism remains unclear in the study, as it remains unclear where the defects are

impacting the SI TRM. This is somewhat addressed by the data in 2a, but 2b/2c argue for a broader impact of Srebp2 on all T cells, regardless of compartment. This discrepancy leaves open the possibility that Srebp2 has multiple roles in the biology of T cells, impacting T cell expansion, effector functon(?), tissue residence, etc. Some clarity is necessary for the paper to have a cohesive message.

We have addressed this point extensively throughout previous points to the other three referees, and is summarized in our response to the comment below.

*However, **the largest conceptual issue with the manuscript is that figure 4 seems unrelated to figures 1-3.** The authors are clearly trying to demonstrate that the deficiency in SI TRM is meaningful, but would be better served by testing a challenge that SI TRM are involved in directly, or focusing on the role of Srebp2 and the pathway described in skin TRM, with a challenge into skin that already contained wt or Srebp2 KO TRM. Without these more direct connections, the paper seems to be lacking in a cohesive message and is probably not ready for publication.*

We have addressed this extensively in the revised version of our manuscript. We now provide new evidence of the cellular effect of manipulating the mevalonate/cholesterol synthesis pathway on the mitochondrial respiration capacity of CD8 T cells and the underlying mechanism (**new Fig. 4**). We have also added new data further reinforcing that T_{RM} and TIL have a common, increased requirement for non-steroidal products of the mevalonate/cholesterol synthesis pathway, and share this underlying mechanism controlling *in vivo* persistence (**new Fig. 5**). Finally. To test an immune challenge demonstrating SI T_{RM} are directly involved in protection, we now have added new data showing that Scap KO are less protected against LCMV rechallenge in the SI than WT mice (**new Fig. 2e and Referee Figure 15**).



Referee Figure 15. LCMV titers by qPCR in the SI of WT and Scap KO mice 3 days after rechallenging with LCMV. NP, nucleoprotein. GP, glycoprotein. Individual replicates shown. Mann-Whitney U Test. *P<0.05

Additional points:

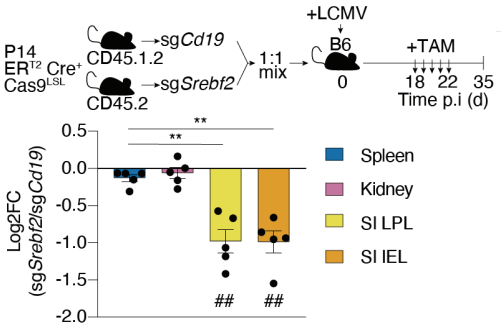
1) Line 136-137, figure 2a:

*Authors showed the change of P14 number in different tissues, but **it's not shown whether these P14 are tissue memory T cells**. They claimed that "Resulted in impaired TRM formation in the SI, but not kidney, WAT, or liver", even though these P14 are TRM. Why? The reduced TRM may be caused by impaired formation of TRM, or reduced formation of effector T cells (since the source of Trm is not clear). Thus, the authors should use more careful time course analysis to better enumerate the numbers of each type of population (effector vs. TRM) in the tissues at different timepoints. Transfer experiments of equal numbers of WT and Srebp2 KO effector CD8 T cells with subsequent analysis of TRM formation would be the most direct means to address the question.*

We realized the source of this confusion might arise from generally referring to IV$^-$ P14 CD8 T cells from tissues at effector time points, ie day 7, as T_{RM}, when in fact, at this time point, these cells are mostly effector CD8 T cells. We have now ensured that these cells are labeled as "IV$^-$ P14 CD8 T cells" when referring to these populations at effector time points.

As the referee mentions, the direct progenitor of T_{RM} is not clear, but they likely arise predominantly from KLRG1^{low} P14 CD8 T cells that seed the tissue between day 4.5-7 after LCMV infection (Milner et al., 2017 Nature). We would like to note that the differences in the SI at day 7 were significantly smaller compared to memory time points, while there were no differences in the spleen at any time point examined (when using Srebf2 KD), including populations of KLRG1low CD127high (memory precursors in the spleen (**new Fig. 2a and Extended Data Fig. 3b**). These data suggest the deficiency in T_{RM} formation upon Srebf2 KD starts after the effector phase, and it is later accentuated as these cells acclimate to tissue residency. To further clarify this point, we have performed an inducible deletion of Srebf2 in a mixed transfer setting in the context of LCMV infection, which was induced after T_{RM} have been established (**Referee Figure 16**). These new data showed that Srebf2 is important for maintaining SI T_{RM}, both IEL and LPL, but not spleen P14 CD8 T cells, nor kidney IV$^-$ P14 CD8

T cells.



CD8 T cells in indicated tissues after inducible deletion of Srebf2 by CRISPR/Cas9 at an early memory time point (day 18-22 after LCMV infection). Data are mean +/- s.e.m. Paired t-Test. **P<0.01. One-sample t-Test. ##P<0.01

2) Line249-250:

The relation of SI Trm's acclimation to niche and the requirement of non-steroidal products like ubiquinone is not shown. Perhaps this is known in the field, but the mechanism should be verified by altering the level of non-steroidal metabolites (i.e., provide the T cells with additional ubiquinones, or OE ubiquinones synthesis enzymes to rescue the defect in Trm formation).

We would like to thank this reviewer for this very insightful comment. Our new data showed that the main cellular effect of limiting the mevalonate/cholesterol synthesis pathway is the reduction of mitochondrial respiration (**new Fig. 4 and Referee Figure 4**). Conversely, we have found that increased mitochondrial respiration due to Fdft1 deletion depends on Pdss2 expression. Importantly, Pdss2 OE alone was able to increase mitochondrial respiration and ubiquinone species production in CD8 T cells. This regulation mechanism applies in the context of infection as shown by our epistatic screen in **Fig. 3d**, and in the context of tumors, where the increased accumulation of Fdft1 KO CD8 T cells intratumorally is reverted by concomitant Pdss2 deletion (new **Fig. 5f and Referee Figure 14**). Furthermore, Pdss2 OE increases CD8 T cell accumulation in tumors, enhances antitumor responses compared to control CD8 T cells, and promotes an increase in the frequency of T_{RM}-like TIL (**new Fig. 5g,j,k and Referee Figure 17**). Combined, these new data strongly reinforce the relation of SI T_{RM}'s acclimation to the niche and the requirement for non-steroidal products. The reliance on particular types of ubiquinones could not be further explored, as we currently lack the technical approaches to deliver titrated amounts of different types of ubiquinone species to the right cellular compartments. Mostly due to the highly hydrophobic nature of ubiquinone, we could not find a valid strategy to provide exogenous ubiquinone during our Seahorse measurements or in vivo experiments.

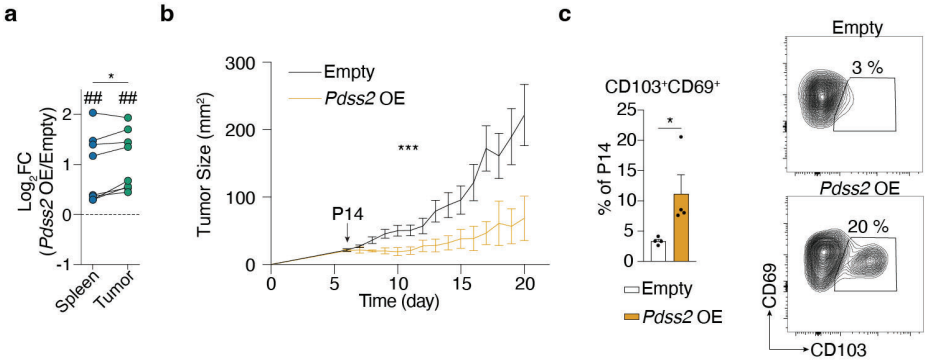


Figure 17. **a**, Log$_2$FC of Pdss2 OE vs Empty P14 CD8 T cells in MC38-GP$_{33-41}$ tumors 5-7 days after adoptive cell transfer. **b**, B16-GP$_{33-41}$ tumor growth curves in mice receiving Empty or *Pdss2* OE P14 CD8 T cells. **c**, Frequency of CD103$^+$CD69$^+$ cells in Empty and Pdss2 OE P14 CD8 T cells in MC38-GP$_{33-41}$ tumors 5 days after adoptive cell transfer. Data are individual replicates (**a**), and mean +/- s.e.m (**b**,**c**) and representative (**c**) or pooled (**a**,**b**) from two independent experiments for a total of n=8 (**a**), n = 9-7 (**b**) and n=13 (**c**). Paired t-Test (**a**,**c**), and two-way ANOVA (**b**). *P<0.05,

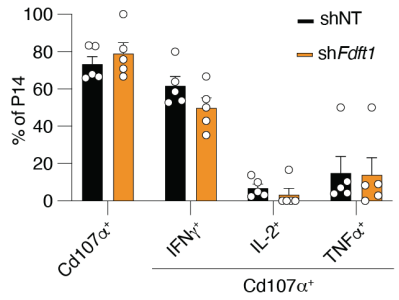
3) Figure 4,

The authors showed increased representation of Ftfd1 KO P14 in tumor and better tumor control, but the mechanism remains unclear.

Our new data included in Fig. 5 shows that increased accumulation of Fdft1 KO CD8 T cells intratumorally is reverted by concomitant Pdss2 deletion (new **Fig. 5f**), suggesting that ubiquinone synthesis is required for the gain of function endowed by Fdft1 loss.

4) The authors do not show functional analyses on the differences in TIL between WT and Ftfd1 KO and determine why the tumors are smaller.

We have assessed Fdft1 KO CD8 T cells functionally by ex vivo re-stimulation with GP_{33-41} peptide. Our data show no difference in the capacity of Fdft1 KO cells to produce cytokines IL-2, TNFα, and IFNγ, or degranulate (Cd107α) on a per-cell basis (**Referee Figure 18**). We politely request not to include these data in the present study to avoid crowding the figures and lengthening the manuscript. Given the lack of functional differences, we believe the increased antitumor efficacy of Fdft1 KO cells arises from their higher capacity to accumulate in the spleen, dLN and tumors, a feature almost universally linked to better tumor control. Of note, Fdft1 deficient TIL had similar expression of exhaustion-associated markers TIM3 and PD1 (new **Extended Data Fig. 7g,h**).



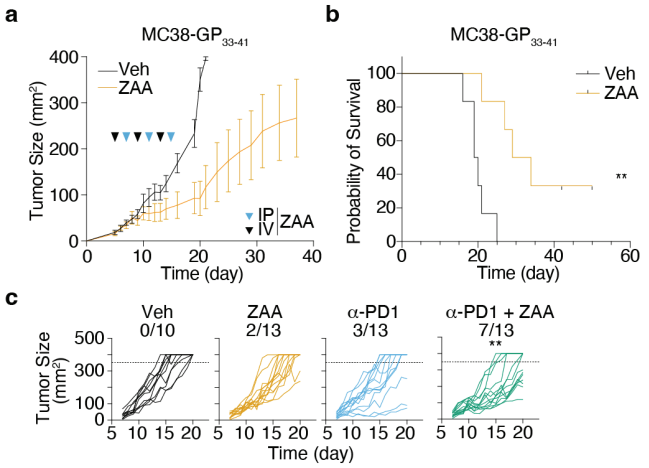
Referee Figure 18. Ex vivo restimulation capacity of WT and Fdft1 deficient P14 TIL upon treatment with GP_{33-41} peptide. Representative results of two independent experiments. Data are

5) More to the point, with ZAA (Ftfd1 inhibitor), this could act on the T cells or tumor cells or other cells in the tumor.

While it has been shown that the antitumor potential of ZAA relies on a competent immune system (Lanterna et al 2016, Cancer Immunol. Immunother.), we acknowledge that our current experiments do not show that ZAA acts specifically through manipulating the cholesterol/mevalonate synthesis pathway in CD8 T cells. We included these promising results using ZAA as a proof of concept and highlight the potential clinical translation with an available Fdft1 inhibitor. Our genetic experiments provide the definitive proof of T cell intrinsic inhibition of Ftft1. We have modified the text accordingly to clarify this point, and we could remove these experiments if the referee considers it necessary.

6) Moreover, ZAA will increase ubiquinone accumulation, and there is a concern regarding which TIL subpopulation is impacted. Is this really a TRM population or expansion of effector T cells? Is there even a ZAA-sensitive population in the tumor and is this a TRM? An alternative explanation is that because ubiquinone is important for the mitochondrial electronic transport chain, and exhausted T cells have mitochondrial dysfunction, it is possible that the ZAA inhibitor is impacting the process of exhaustion, not altering TRM. Likewise, it could impact effector function and increase the potency of effector T cells in tumors. Or ZAA could enhance migration into the tumor tissue (from the margin), or into the tissue in general. Or ZAA could increase proliferation of T cells in the tumor, which would allow them to better compete with the highly proliferative tumor cells reduce tumor growth. On their own, these would be interesting phenomena, but they would not be directly related to the data in Figs 1-3. The authors need to show whether these are distinct or similar phenomena and determine how the ZAA impacts the T cells vs. tumor cells.

These questions are extremely interesting but are beyond the scope of our current study. We have modified the text to clarify our experiments with ZAA are meant as a proof of concept that shows a potential promising clinical applicability of inhibiting Fdft1 alone (**new Extended Data Fig. 7i,j and Referee Figure 19a,b**) or in combination with anti-PD1 therapy (**new Fig. 5l Referee Figure 19c**). The mechanistic basis for this interesting effect will be addressed in subsequent studies. Thus, we have also removed the data showing increased ubiquinone production in CD8 T cells treated with ZAA.



ɛe Figure 19. **a**, MC38-GP$_{33-41}$ tumor growth curves of mice receiving ZAA or vehicle. **b**, Survival of mice implanted with MC38-GP$_{33-41}$ tumors receiving ZAA or vehicle. **c**, B16-GP$_{33-41}$ tumor growth curves of mice receiving vehicle control, ZAA, αPD-1, or ZAA and αPD-1 Ab. ɪk test (**b**), Fiser's exact test (**c**). **P<0.01

4. The title of the work is confusing, as it suggests metabolic regulation is important for tissue memory T cell formation/maintenance/migration and that this is relevant for TIL. However, the case best made for SI TRM and cholesterol. It is not certain that there are TRM in the skin that rely on this pathway or that the phenomenon in tumors is related to the phenomenon in the SI TRM. The best data shown is that Ftdt1 inhibition/ko leads to smaller tumors, but how this is achieved is not clear. Specifically, why would the formation/maintenance/ migration of TRM into tumor be related to smaller tumors (as opposed to increased effector function), and if the mechanism is increased effector function, is that the same mechanisms maintaining/forming/migrating TRM into the SI? Seems likely to be at least two distinct phenomena, and that this metabolic pathway is important for many aspects of T cell biology.

We do not intend to say T_{RM} and TIL share all metabolic adaptations. Our data show that T_{RM} and functional TIL share common metabolic requirements to provide effector function and accumulate in non-lymphoid environments and, thus are poised to respond similarly to enhancing these adaptations, such as Pdss2 OE. We believe our extensively revised manuscript clarifies and supports this novel idea and provides exciting new strategies to enhance immunity in tissues and anti-tumor responses.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

This revised manuscript is much improved and clearer. The narrative has been has been focused and the experiments more aimed at the role of ubiquinone to maintain T cell mito respiration and Trm formation are more convincing. There are just a few additional points with the new data that the authors should look at to firm up some of their conclusions.

1) it would be important for the authors to explore the model that increased cholesterol suppresses ubiquinone synthesis more directly, which is contributing to the toxicity of the Trm cells and loss of OXPHOS. A few things the authors could try is to take the T cells out of the HC diet and then give them ubiquinol or examine if treatment of T cells with physioloigcal amounts of cholesterol in vitro (or LDL) reduces ubiquinone levels as shown in Fig 4F/G and mitoOXPHOS by Seahorse.
2) ANother alternative would be to feed mice ubiquinol and HC diets to see if the ubiquinol can rescue HC diet. This again would be of greater impact for obesity-related health issues.
2) it would be nice to show the mito mass and potential staining in the TILs with Pdss2 OE to see if this is rescuing the TILs mito function in vivo too.
3) It would be important for teh ZAA treatments + anti-Pd-1 in MC38 tumors to be conducted with CD8 T cell depeltion to show that the therapeutic effects are T cell dependent and not simply because of an effect of ZAA on tumors.
4) Lastly, the response to point #4 from Rev 1 is unsatisfactory. It's a minor point, but the authors have the data collected on the total liver Trm cells as most of those will be iv positive and those numbers should be used in the calculations. What is the fraction of the total liver Trm that are iv-? It has to be a small percent if the labeling was done correctly.

Referee #2 (Remarks to the Author):

The authors have generally responded well to my concerns.

However, I do not agree with the fact that the authors focus on metabolic adaptations, have lipidomics data but almost solely focus on transcriptomics adaptations without laying the necessary links between a gene transcript and its actual functional effectors, the respective enzymes (proteins) and metabolites. This also extends to the fact that lipid composition of cells is becoming increasingly recognized as important cell faith decision modulator. What then confuses me is that the authors have metabolomics and lipidomics data but no proteomics data? Moreover, the authors argue that they would need 600 mice for a Western plot, fair point not practical, but if WB is out of question, why did they not do a proteomics analysis? That should be feasible with the 10k cells the authors can obtain and could hence have been done at their core facility.

Finally, as earlier indicated a very valuable data set, but a better integration of transcriptome, proteome and metabolome would have been highly appreciated. Don't misunderstand me, very

sound study but I just feel that integrating all layers would have sketched an even more comprehensive picture.

Referee #3 (Remarks to the Author):

The authors have made a good response to the critiques and linked the SI and tumour parts better. I think they should include the functional data in Referee fig 18 - even if it is negative it is useful. It is still not clear how the effect is mediated at this point.

I still had a couple of concerns.

The statin experiments are very condensed. The LCMV experiment could be better displayed as it would be better to see the underlying data rather than just ratios. More importantly it looked like the spleen responses were affected by the statin treatment, but the way it is written it sounds like just the Trm are affected. It could be quite a different effect being manifest by statins here. It has been known since the 1980s that statins can impact on lymphocyte function, and eg recently effects on other T cell subsets have been shown (https://pubmed.ncbi.nlm.nih.gov/36332451/). The specificity of this effect is therefore unclear.

Also for the human study - which is quite hard to understand (NB it says spleen in the text, but it is a study of PBMCs), it wasn't clear why a score (signature) was given, rather than counting the relevant subsets in the dataset since it is scRNAseq. Anyway, as far as I can tell the study referenced is of ulcerative colitis and was taking rectal biopsies not SI cells. If there were additional SI biopsies also taken a lot more data would need to be included to understand that, and if they are indeed rectal biopsies this is not an accurate part of the rebuttal.

Referee #4 (Remarks to the Author):

Major point: I think the paper needs to be streamlined and redo the main section in line with below. The data supporting the comparing TRM to other T cells is rather weak because of inconsistency, but framing this as an investigation of a metabolic pathway in SI TRM and then using the info to improve tumor responses is much stronger.

I had a very negative view of the paper at first and THIS IS ENTIRELY A PROBLEM WITH THE ABSTRACT and MAIN SECTIONs, as the discussion is fine. I started writing this while reading the Main section and kept so that it can help the authors to see the issues with the current version:

What is the common thread here? There are too many inconsistencies and too much lack of clarity of what is being shown to draw the strong conclusions. Take figure 1: A-C compare TRM from liver and SI and other memory subsets considered “circulating” and show knockout data and metabolic signatures that show SI TRM are distinct from other subsets for Melvonate/Cholesterol and OXPHOS

complex II. Then they switch to Kidney vs. SI TRM for metabolites and data in F and G for Melvonate/Cholesterol score that shows Kidney cells have high score at day 7 (effector) and then low score at day 35 (memory) (F) and then (G) that day 30 neither liver nor kidney TRM are high for this score, but SI is. SI also has Srebf2, but kidney and liver do not (including IF). All of this would indicate that SI TRM alone have upregulation of the Melvonate/Cholesterol pathway, but the impact of metabolic pathways for other TRM is not well shown at all.

1. This makes this conclusion a reach: "Together, these data revealed that TRM possessed a distinct metabolic program compared to their circulatory counterparts…" this was not really shown. There's not even statistics to suggest this in 1F. What are the pathways that distinguish Liver TRM from circulating? What are the pathways that distinguish kidney TRM from circulating? The only data is that SI TRM are different and specifically that they express Srebf2 and TRM in kidney and other locations do not.
2. Fig 2 completely changes the story and interpretation of the data. Now knockdown with shSrebf2 only impacts SI TRM in LCMV, but knockdown with shSrebf2 impacts all T cells in listeria, and knockout with sgSrebf2 in LCMV affects all T cells. This includes Kidney TRM. Didn't you just show the protein isn't expressed in Kidney TRM, and now the knockout has an effect??
3. Maybe this could have been explained by the original data in 1b, as this was a CRISPR screen. That data (in Table S1 and Fig 3A) shows that Srebf2 knockout has an effect in all cells but is strongest in SI TRM. The color scale in Fig 3A hides this a bit.
4. Lova has effects in spleen memory and kidney (again). What is the message?

So what is the "common metabolic adaptation that empowers CD8 T cell residency"? Is it the melvonate pathway? Because that is involved in many T cell subtypes (TEM, TCM, Kidney TRM/Liver TRM), depending on the assay and time point. It also is (and is not) important for TRM in Kidney and Liver (again depending on assay). The inconsistencies and over-interpretation make it feel like the authors are trying to make the data fit into a preconceived storyline as opposed to trying to interpret they are getting and determine what the data mean in aggregate, to put it together. I do see a way forward, but the manuscript currently is very unclear and must have a clear message. The data are inconsistent in the other tissues and why this is seems random. Maybe just make the story focus only on SI TRM, which give clear and consistent data, rather than the comparison between T cells that circulate or are in other tissues (where they sometimes depend on the pathways and/or genes involved and sometimes do not).

After reading discussion: I think the paper might benefit from just removing the "data interpretation" from the main section (which is often confusing and over-interpreted) and just rely on the conclusions made in the discussion (which are grounded in the data and much more measured). It might even benefit the story to move some of the data that is inconsistent regarding TRM in other tissues to supplement and acknowledge that SI TRM are a clear picture and the picture outside of this site are less consistent. I do not think it is clear that this is a difference between SI TRM vs. other cells or TRM vs. circulating T cells. It is just clear that these pathways are important in SI TRM and there are some real-world implications vis a vis diet and drug therapy on SI TRM. I think in this context, using the information gleaned from SI TRM to inform tumor therapy makes sense (the connection to tumor T cells makes more sense as a therapeutic question, which is how it is presented.

To this point, it would help to repeat 2D/E with lovastatin pretreated mice (i.e., LCMV, lovastatin, then infection) to see if the protection by SI TRM is impaired.

Minor point:

P14 recognizes Db, not Kb (line 67).

Author Rebuttals to First Revision:

Common Metabolic Adaptations Empower CD8 T Cell Tissue Residency and Antitumor Immunity

Reina-Campos et al.

Point by Point Response to Referees v2

We thank the referees for their continued constructive feedback and guidance. We are delighted that all referees agree that our study has been much improved and is now clearer, more focused, and offers a better and stronger narrative linking the study of T_{RM} in the context of infection as an avenue to enhancing antitumor CD8 T cell responses. We have addressed the remaining points to reinforce our conclusions, improve our figure display, and improve clarity. Ultimately, this study has focused on one key aspect of the metabolic adaptations of T_{RM} supported by metabolomics, functional screens, and pharmacologic, dietary, and genetic perturbations and has offered new strategies for enhancing functional antitumor immunity.

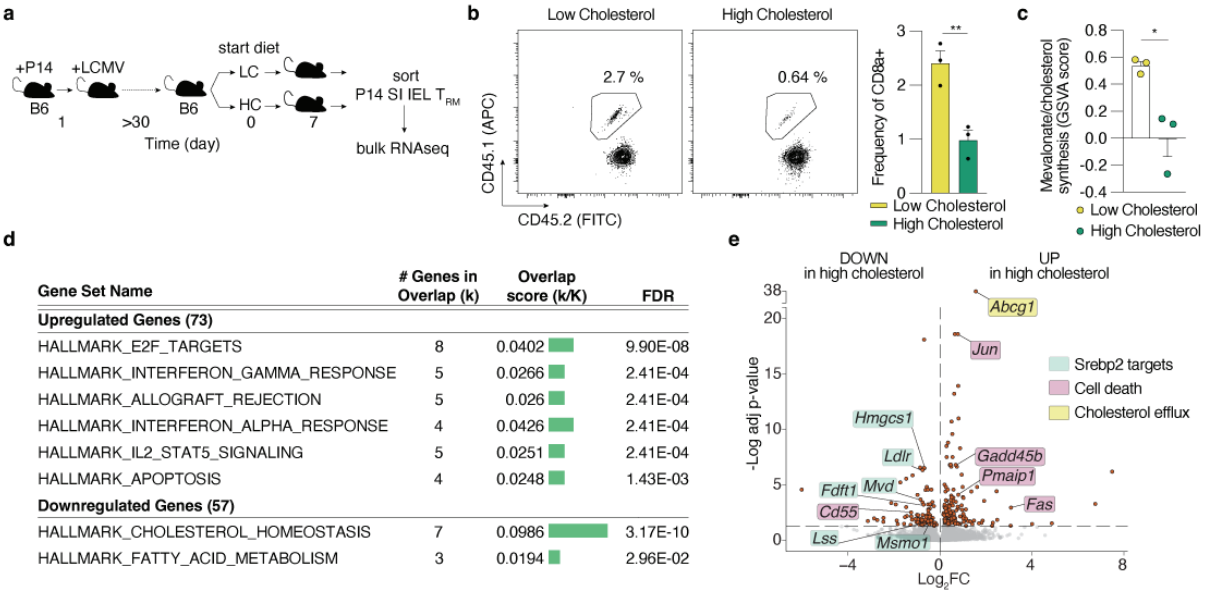
The key additions include: a better understanding of the toxicities of high cholesterol diet on SI T_{RM}, and how it can be rescued by *Pdss2* OE, a deeper understanding of how statins impact CD8 T cell respiration, a reanalysis of liver T_{RM} populations, and a better understanding of the requirements of CD8 T cells for the anti-tumoral effect of ZAA. We believe our study has improved substantially as a result. Please, find our point-by-point response below:

Referee #1:

This revised manuscript is much improved and clearer. The narrative has been has been focused and the experiments more aimed at the role of ubiquinone to maintain T cell mito respiration and Trm formation are more convincing. There are just a few additional points with the new data that the authors should look at to firm up some of their conclusions.

1) it would be important for the authors to explore the model that increased cholesterol suppresses ubiquinone synthesis more directly, which is contributing to the toxicity of the Trm cells and loss of OXPHOS. A few things the authors could try is to take the T cells out of the HC diet and then give them ubiquinol or examine if treatment of T cells with physioloigcal amounts of cholesterol in vitro (or LDL) reduces ubiquinone levels as shown in Fig 4F/G and mitoOXPHOS by Seahorse. ANother alternative would be to feed mice ubiquinol and HC diets to see if the ubiquinol can rescue HC diet. This again would be of greater impact for obesity-related health issues.

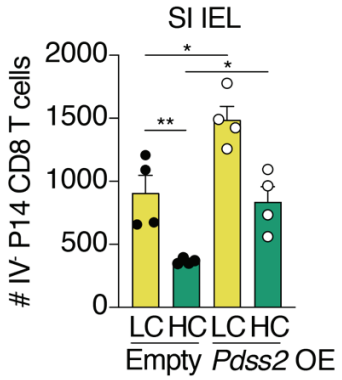
We thank the referee for their suggestions. We have further explored the underlying toxicity of excess dietary cholesterol on SI T_{RM} cells by performing ultra-low input RNAseq analysis of established SI T_{RM} cells (IV^- P14 CD8 T cells from the intraepithelial compartment) from mice fed high or low cholesterol-containing diets for 7 days (**new Extended Data Fig. 5e and Referee Figure 1a**). Of note, 7 days of dietary treatment had a functional impact on established SI T_{RM} cells (**new Extended Data Fig. 5f and Referee Figure 1b),** and markedly suppressed the mevalonate/cholesterol synthesis pathway (**new Extended Data Fig. 5j,k,l and Referee Figure 1c,d,e)**. We did not observe differences in tissue-retention molecules, nor a decrease in T_{RM} core genes among the 142 differentially expressed genes, suggesting T cell differentiation was not impacted at this time point. On the other hand, several molecules associated with the induction of cell death, such as *Fas*, were upregulated up to 4-9 times in SI T_{RM} cells from mice fed a high vs a low cholesterol-containing diet (**new Extended Data Fig. 5l and Referee Figure 1d,e).**



Gene Set Name	# Genes in Overlap (k)	Overlap score (k/K)	FDR
Upregulated Genes (73)			
HALLMARK_E2F_TARGETS	8	0.0402	9.90E-08
HALLMARK_INTERFERON_GAMMA_RESPONSE	5	0.0266	2.41E-04
HALLMARK_ALLOGRAFT_REJECTION	5	0.026	2.41E-04
HALLMARK_INTERFERON_ALPHA_RESPONSE	4	0.0426	2.41E-04
HALLMARK_IL2_STAT5_SIGNALING	5	0.0251	2.41E-04
HALLMARK_APOPTOSIS	4	0.0248	1.43E-03
Downregulated Genes (57)			
HALLMARK_CHOLESTEROL_HOMEOSTASIS	7	0.0986	3.17E-10
HALLMARK_FATTY_ACID_METABOLISM	3	0.0194	2.96E-02

Referee Figure 1. Transcriptional effect of acute excess of dietary cholesterol on SI T_{RM} populations. **a**, Experimental design to measure transcriptional changes induced by dietary cholesterol on established SI T_{RM} by ULI-RNAseq, **b**, SI T_{RM} frequencies after 7 days of dietary intervention. **c**, Mevalonate/cholesterol synthesis GSVA scores in SI T_{RM} from mice fed a high vs low cholesterol-containing diet. **d**, Gene pathway analysis of upregulated and downregulated genes between SI T_{RM} from mice fed a high vs low cholesterol-containing diet (MsigDb, Hallmark gene sets). **e**, Volcano plot of differentially expressed genes in SI T_{RM} from mice fed a high vs a low cholesterol-containing diet. Un-paired t-Test (**b**, **c**). *P<0.05, **P<0.01.

Given that ubiquinone species are among the most hydrophobic lipid species, we, and others in the field, have been unable to deliver these in vitro or in vivo, which prevents us from performing ubiquinone add-back rescue experiments. However, given that Pdss2 OE increases mitochondrial respiration and ubiquinone production (**Fig. 4**), we tested whether Pdss2 OE could offset the toxicities of excess dietary cholesterol. Indeed, *Pdss2* OE P14 CD8 T cells rescue SI T_{RM} formation to WT levels in mice fed a high cholesterol diet (**new Figure 3f and Referee Figure 2**). Of note, a low-cholesterol environment further promotes SI T_{RM} formation as, under this condition, we expect the Srebp2-driven pathway to be highly active and further fuel the Pdss2 branch. Combined, these data suggest that excess dietary cholesterol likely reduces the mitochondrial respiratory capacity of CD8 T cells, which then makes them more susceptible to cell death, as suggested by *Cd55* downregulation or *Fas* upregulation, a process that can be partially offset by enforcing the production of ubiquinone by *Pdss2* OE.



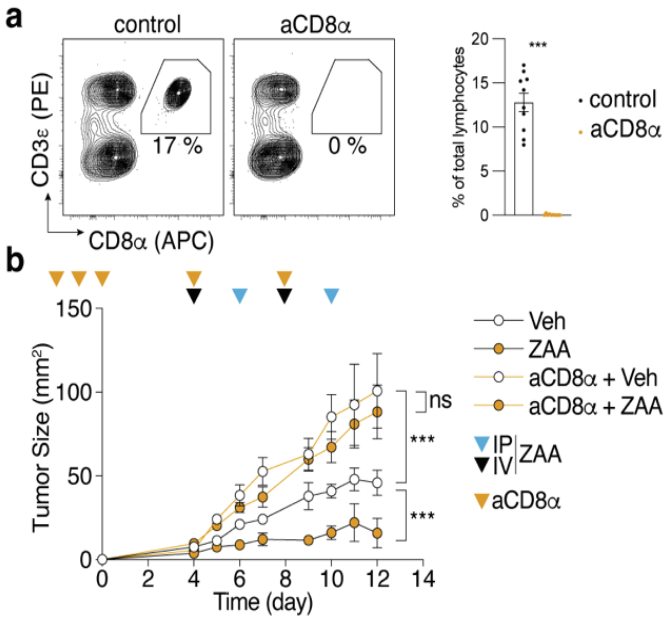
Referee Figure 2. Total cell numbers of Empty and *Pdss2* OE SI IEL P14 CD8 T cells in mice fed low or high cholesterol-containing diets. Un-paired t-Test. *P<0.05, **P<0.01.

2) it would be nice to show the mito mass and potential staining in the TILs with Pdss2 OE to see if this is rescuing the TILs mito function in vivo too.

We and others have found that mitochondrial mass and mitochondrial potential stainings have limitations related to how these dyes react with the specific lipidic composition of the mitochondria of T_{RM} (Konjar et al 2018, Science Immunology), which could confound results in T_{RM}-like TIL as well. For ex vivo analysis, we are limited by the cellular recovery of Pdss2 OE CD8 T cells from tumors, which prevent us from performing respirometry analysis in these populations. We believe our Seahorse analysis of genetically manipulated CD8 T cells is the best approach to showing how Pdss2 enhances mitochondrial respiration (**Fig. 4**).

3) It would be important for teh ZAA treatments + anti-Pd-1 in MC38 tumors to be conducted with CD8 T cell depeltion to show that the therapeutic effects are T cell dependent and not simply because of an effect of ZAA on tumors.

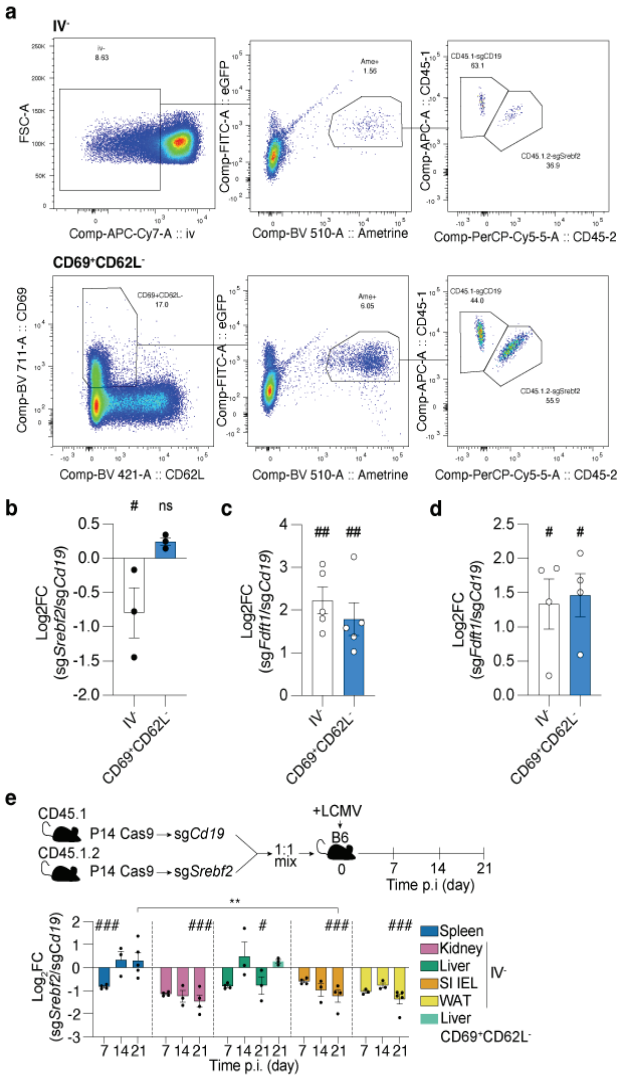
We have included new data showing that the anti-tumoral effect of ZAA on MC38-GP_{33-41} is abolished in the absence of CD8 T cells (**new Extended Data Fig. 7m and Referee Figure 3**). These data align well with previous reports showing that an intact immune system is required for the antitumor actions of ZAA in mice (Lanterna et al 2016, Fig. 1d, PMID: 27520505).



blood of mice treated with aCD8a Ab at the time of tumor inoculation. **b,** MC38-GP_{33-41} tumor growth curves of mice receiving ZAA in combination with CD8 T cell depletion. Un-paired t-Test (**a**). Two-way ANOVA (**b**). ***P< 0.005.

4) Lastly, the response to point #4 from Rev 1 is unsatisfactory. It's a minor point, but the authors have the data collected on the total liver Trm cells as most of those will be iv positive and those numbers should be used in the calculations. What is the fraction of the total liver Trm that are iv-? It has to be a small percent if the labeling was done correctly.

We thank the referee for noting this and are sorry we did not understand the point previously. We have reanalyzed the liver data for the Srebp2 deletion experiment (**Fig. 2b**) according to the suggested gating strategy (**Referee Figure 4a**). The relatively small differences observed in liver T_{RM} (based on IV stain) upon Srebp2 KO are absent when cells are gated on the $CD69^{+}CD62L^{-}$ population (**Referee Figure 4b**). These data suggest that this T_{RM} population is the least sensitive to Srebp2 activity, which is in line with our CRISPR screen, statin, and SCAP KO data. For the gain of function phenotypes observed upon Fdft1 deletion and Pdss2 OE, we see consistent results independent of the gating strategy of P14 CD8 T cells in the liver (**Referee Figure 4c,d**). We have added this data to the new Fig. 2b and modified the text accordingly (**Referee Figure 4e**).



strategies to define liver T_{RM} populations, **b,** Relative frequencies of P14 CD8 T cell populations as calculated by two different gating strategies in the Srebp2 KO experiment at day 21 pi, as related to Fig. 2b. **c**, Relative frequencies of P14 CD8 T cell populations as calculated by two different gating strategies in the Fdft1 KO experiment at day 14 pi, as related to Fig. 3c. **d**, Relative frequencies of P14 CD8 T cell populations as calculated by two different gating strategies in the Pdss2 OE experiment at day 14 pi, as related to Fig. 3e. **e**, final Figure 2b, including values for the additional liver T_{RM} gating strategy based on the expression of CD69 and CD62L markers. One-sample t-Test (**b**, **c**, **d**). #P< 0.05, ##P<0.01.

Referee #2:

The authors have generally responded well to my concerns.

However, I do not agree with the fact that the authors focus on metabolic adaptations, have lipidomics data but almost solely focus on transcriptomics adaptations without laying the necessary links between a gene transcript and its actual functional effectors, the respective enzymes (proteins) and metabolites. This also extends to the fact that lipid composition of cells is becoming increasingly recognized as important cell faith decision modulator. What then confuses me is that the authors have metabolomics and lipidomics data but no proteomics data? Moreover, the authors argue that they would need 600 mice for a Western plot, fair point not practical, but if WB is out of question, why did they not do a proteomics analysis? That should be feasible with the 10k cells the authors can obtain and could hence have been done at their core facility.

Finally, as earlier indicated a very valuable data set, but a better integration of transcriptome, proteome and metabolome would have been highly appreciated. Don't misunderstand me, very sound study but I just feel that integrating all layers would have sketched an even more comprehensive picture.

We thank this referee for noting our very valuable data set. We hope our web-based application (FIT.db) will serve to better visualize our results in the context of other datasets. Integration of these many datasets is not trivial, and this current study has focused on one key aspect of the metabolic adaptations of T_{RM}

supported by metabolomics, functional screens, as well as pharmacologic, dietary, and genetic perturbations. It is our hope that these extensive datasets will be of value to the field and will be more rapidly integrated into the broader understanding of T cell fate than if they remain unpublished. Regarding low cell proteomics—we have started to pursue this angle but find the relevant enzymes are not well detected at low cell input with the current analyses we have access at the moment, preventing comparisons to our other datasets.

Referee #3:

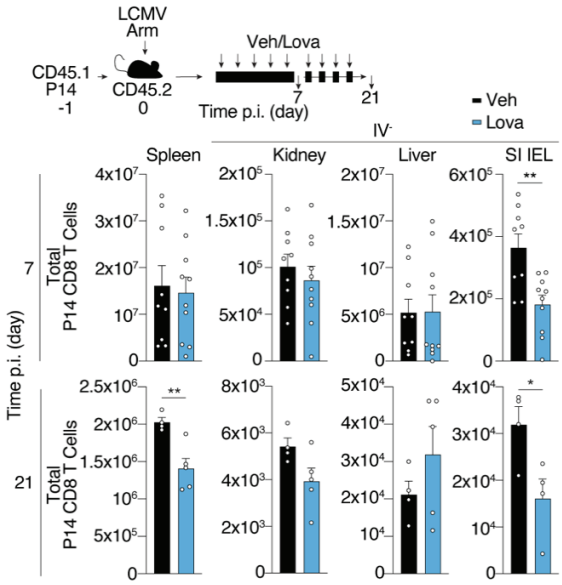
The authors have made a good response to the critiques and linked the SI and tumour parts better. I think they should include the functional data in Referee fig 18 - even if it is negative it is useful. It is still not clear how the effect is mediated at this point.

We have now included the ex vivo re-stimulation data (**new Extended Data Fig. 7i,j**)

I still had a couple of concerns.

The statin experiments are very condensed. The LCMV experiment could be better displayed as it would be better to see the underlying data rather than just ratios.

We have modified the figure to display total cell numbers for each tissue and timepoint (**new Fig. 2f and Referee Figure 5**).



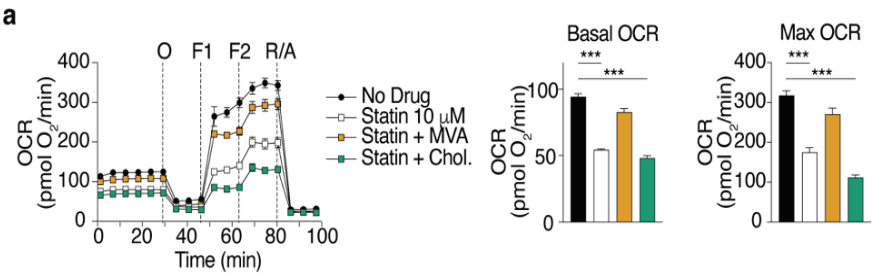
Referee Figure 5. Quantification of total P14 CD8 T cells from indicated tissues at day 7 and 21 after LCMV infection in mice treated with vehicle or Lovastatin (Lova) as indicated. Un-paired t-Test. *P<0.05, **P<0.01.

More importantly it looked like the spleen responses were affected by the statin treatment, but the way it is written it sounds like just the Trm are affected.

It could be quite a different effect being manifest by statins here. It has been known since the 1980s that statins can impact on lymphocyte function, and eg recently effects on other T cell subsets have been shown (https://pubmed.ncbi.nlm.nih.gov/36332451/). The specificity of this effect is therefore unclear.

We have modified the text to acknowledge the defect observed on spleen P14 CD8 T cells and discussed these results in the context of the known immunomodulatory effects of statins. We agree with the reviewer that statins might exert negative effects at multiple levels, and we have modified the text to acknowledge this. The statin data serves to complement the genetic data exploring the role of this pathway in a T cell intrinsic manner.

In addition, we provide new data that shows how statin treatment diminishes mitochondrial respiration of CD8 T cells in a mevalonate-dependent, but cholesterol-independent manner (**new Fig. 4b and Referee Figure 6**). Thus, while statins might have a broad effect, ie cellular proliferation due to lower cholesterol production output similar to the loss of SCAP (Kidani et al., 2013 Nat. Imm.), the effect seen on the respiratory capacity of CD8 T cells under statin treatment depends on non-steroidal metabolites.



Referee Figure 6. Oxygen consumption rate (OCR) of in vitro activated CD8 T cells treated with statin (simvastatin) in combination with mevalonolactone (MVA) or cholesterol (Chol.) and subjected to the MitoStress test (Seahorse). Un-paired t-Test ***P<0.005.

Also for the human study - which is quite hard to understand (NB it says spleen in the text, but it is a study of PBMCs), it wasn't clear why a score (signature) was given, rather than counting the relevant subsets in the dataset since it is scRNAseq. Anyway, as far as I can tell the study referenced is of ulcerative colitis and was taking rectal biopsies not SI cells. If there were additional SI biopsies also taken a lot more data would need to be included to understand that, and if they are indeed rectal biopsies this is not an accurate part of the rebuttal.

We thank the referee for noting this. The "spleen" label in the figure legend is a typo and has now been corrected to "blood" as these were indeed PBMC samples. To clarify: the original study by Boland et al 2020 did not include SI samples, but SI samples from healthy and UC patients were indeed collected in the course of the same study. Only samples from healthy donors have been included in our study. The raw data for these samples will be published as part of our study as they also relate to Supplementary Data Figure 2e, and will be deposited to GEO under GSE207044. The lack of antigen-specificity or clonotype for the CD8 T cells profiled in this study makes the interpretation of frequencies very challenging, and thus we opted to focus on a widely used (Milner et al., 2017 Nature) tissue-residency gene signature to obtain a qualitative readout of the transcriptional cellular estate of CD8 T cells in blood and SI. We believe our results are potentially of high significance in the human context, and thus of interest to the general public, which will hopefully lead to further investigation.

Referee #4:

Major point: I think the paper needs to be streamlined and redo the main section in line with below. The data supporting the comparing TRM to other T cells is rather weak because of inconsistency, but framing this as an investigation of a metabolic pathway in SI TRM and then using the info to improve tumor responses is much stronger….After reading discussion: I think the paper might benefit from just removing the “data interpretation” from the main section (which is often confusing and over-interpreted) and just rely on the conclusions made in the discussion (which are grounded in the data and much more measured). It might even benefit the story to move some of the data that is inconsistent regarding TRM in other tissues to supplement and acknowledge that SI TRM are a clear picture and the picture outside of this site are less consistent. I do not think it is clear that this is a difference between SI TRM vs. other cells or TRM vs. circulating T cells. It is just clear that these pathways are important in SI TRM and there are some real-world implications vis a vis diet and drug therapy on SI TRM. I think in this context, using the information gleaned from SI TRM to inform tumor therapy makes sense (the connection to tumor T cells makes more sense as a therapeutic question, which is how it is presented.

We thank the referee for noting the real-world implications of our study and for helping us streamline our narrative. We agree with this referee that the SI T_{RM} offer a clear picture. Kidney and liver T_{RM} populations were included to the extent possible (ie not enough kidney T_{RM} for the full metabolic screen), in addition to circulatory populations, to provide context and additional points of reference to understand the effects observed in SI T_{RM}, as we believed T_{RM} populations needed to be compared to other T_{RM} populations, in addition to circulatory memory CD8 T cells. To this end, we chose the liver and kidney as tissue-resident populations with relatively lower Srebp2 activity. In addition, we have employed multiple genetic approaches, a dietary approach, and a pharmacological approach to perturb Srebp2 and the mevalonate/cholesterol synthesis pathway. In line with the role of Srebp2 as an enhancer of this pathway, the complex regulation of Srebp2 by its products, and the nature of *in vivo* modulation (ie dosage, route of delivery), each of these perturbations inhibit the mevalonate/cholesterol synthesis pathway to varying degrees, which are overall hard to capture by one assay, but ultimately reflected by the extent they impair the formation of T_{RM}. We believe this nuance is important and have made every effort to make our interpretations rigorous.

Combined, our data suggest that enhanced activity downstream of Hmgcr, Scap and Srebp2 is a metabolic requirement of CD8 T cells to become SI T_{RM}. We observed graded reliance on Srebp2 among the different tissues; where SI T_{RM} showed a greater dependence on the pathway than kidney, and WAT, followed by liver resident cells, reflecting the pattern of expression for genes involved in this pathway (**new Fig. 1g and new Extended Data Fig. 2d**). Of note, reanalysis of the Srebp2 KO and lovastatin experiments have refined our results that are now more in line with the main message of this study (**new Fig. 2b and 2f and Referees Figure 4 and 5**). We have modified the abstract and main sections of this study to make all these points clearer and present a more cohesive and measured message.

To this point, it would help to repeat 2D/E with lovastatin pretreated mice (i.e., LCMV, lovastatin, then infection) to see if the protection by SI TRM is impaired.

Given the loss of SI T_{RM} accumulation with lovastatin treatment, we expect protection to be impaired.

Minor point:

P14 recognizes Db, not Kb (line 67).

An embarrassing typo—it has now been corrected in the text.

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have responded to my concerns in the revision and the new data added provides stronger evidence for their conclusions and interpretations. I can recommend this study for publication.

Referee #2 (Remarks to the Author):

See my previous assessments this is a 2nd revision.

My opinion that a multi-omics integration would have been highly valuable to this study has not changed. Nevertheless, this is out of the reach for the authors at present as seemingly is proteomics on 10k immune cells, too. Moreover and as I have also indicated multiple times this is none the less a very elegant valuable study/data set and I do not want to stand in the way of its publication.

Referee #3 (Remarks to the Author):

The authors have explained the use of SI data and given some details, but it is still a bit unclear what was included – I'm guessing this was terminal ileum. This might seem like a small point but the authors already show there's a big difference between ileum, duodenum and jejunum.

The authors also write
"Similarly, protein levels of Hmgcr, an Srebp2 target, and the rate-limiting enzyme of the mevalonate/cholesterol synthesis pathway, were also higher in human SI and colonic CD8 T cells compared to those in the spleen (Extended Data Fig. 2f)."
These data are shown in Extended Fig 2g. The referencing is all out of sync here. It is possible to figure out where these tissues came from but is it just one spleen? The numbers are not given.

Referee #4 (Remarks to the Author):

The manuscript is much better. I appreciate the efforts to streamline and think it makes a much clearer story.

Author Rebuttals to Second Revision:

Metabolic Programs of T Cell Tissue Residency Empower Tumor Immunity

Reina-Campos et al,

Point by Point Response to Referees v3

We thank the referees for their expert feedback and guidance in improving our manuscript. We are thrilled that Referees #1, #2 and #4 consider our manuscript ready for publication.

We have addressed the remaining points raised by Referee #3 below.

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have responded to my concerns in the revision and the new data added provides stronger evidence for their conclusions and interpretations. I can recommend this study for publication.

Referee #2 (Remarks to the Author):

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My opinion that a multi-omics integration would have been highly valuable to this study has not changed. Nevertheless, this is out of the reach for the authors at present as seemingly is proteomics on 10k immune cells, too. Moreover and as I have also indicated multiple times this is none the less a very elegant valuable study/data set and I do not want to stand in the way of its publication.

Referee #3 (Remarks to the Author):

The authors have explained the use of SI data and given some details, but it is still a bit unclear what was included – I'm guessing this was terminal ileum. This might seem like a small point but the authors already show there's a big difference between ileum, duodenum and jejunum.

In all mice experiments, the entire small intestine was processed. In the human scRNAseq cohort, small intestine samples were taken from the ileum (GSE125527). We have added this information to their respective methods section.

The authors also write

"Similarly, protein levels of Hmgcr, an Srebp2 target, and the rate-limiting enzyme of the mevalonate/cholesterol synthesis pathway, were also higher in human SI and colonic CD8 T cells compared to those in the spleen (Extended Data Fig. 2f)."

These data are shown in Extended Fig 2g. The referencing is all out of sync here. It is possible to figure out where these tissues came from but is it just one spleen? The numbers are not given.

The figure calling has now been updated to "Extended Data Fig. 2g" and numbers are now included in the figure legend. The data presented in Extended Data Fig. 2g is from one spleen.

Referee #4 (Remarks to the Author):

The manuscript is much better. I appreciate the efforts to streamline and think it makes a much clearer story.