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Peer Review Information

Journal: Nature Immunology
Manuscript Title: Effector differentiation downstream of lineage commitment in ILC1 is driven by Hobit across tissues
Corresponding author name(s): Georg Gasteiger

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A31400
Message: 16th Feb 2021

Dear Dr Gasteiger,

Thank you for providing a point-by-point response to the referees comments on your manuscript entitled, "Effector differentiation downstream of lineage commitment in ILC1 is driven by Hobit across tissues". As noted previously, while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the current manuscript for publication, but would be very interested in considering a revised version along the lines proposed in your response..

We invite you to submit a substantially revised manuscript, however please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

Specifically, the revision should include new experiments to address as summarized in your response:

- compare gene expression patterns of ILCPs with the early Tcf7hi ILC1s that we identified in this manuscript

- include analyses of ILCPs in Hobit reporter and KO mice to further delineate the developmental stage at which Hobit expression is induced

- provide evidence for commitment of the ILC1 lineage using new Hobit fate-mapping analyses

- study in vivo regulators of Hobit expression in the context of ILC1 differentiation

- validate the cytotoxic potential suggested by the gene expression patterns of CD127- Gzm+ ILCs

- include analyses of ILC1 differentiation for requested tissues, particularly the gut and lymphoid organs

- include analyses of ILC1 differentiation in a model of tissue inflammation

Please include the additional textual clarifications as indicated in your response letter.

When you revise your manuscript, please take into account all reviewer and editor comments, please highlight all changes in the manuscript text file in Microsoft Word format.

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

If revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

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

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

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If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Immunology or published elsewhere.

Nature Immunology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

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

Thank you for the opportunity to review your work.

Kind regards,

Laurie

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Referee expertise:

Referee #1: Innate lymphoid cells

Referee #2: Innate immunity

Referee #3: Innate lymphoid cells

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this study Gasteiger and colleagues investigated the putative heterogeneity of liver-resident ILC1s. The authors demonstrate that previously described hepatic IFNg-producing "helper" ILC1s and granzyme and perforin producing "cytotoxic" ILC1s are in fact the same cell type that can present itself in distinct modalities. This study builds on previous work that identified Hobit as an essential regulator of hepatic ILC1 tissue-residency and survival in mice. Here, employing flow cytometric and RNA sequencing techniques the authors demonstrate that the cellular state is determined by the transcriptional regulator Hobit, which pushes "helper" ILC1s towards "cytotoxic" ILC1s. Differentiation towards cytotoxic ILC1s was accompanied by the gradually loss of Kit, Tcf7 and Cd127 expression and (potentially as a consequence of that) loses its potential to proliferate as compared to helper ILC1s. Finally the authors demonstrate that the Hobit-dependent cytotoxicity is not a hepatic ILC1-specific phenomenon, but seems to be conserved in salivary glands and kidney resident innate lymphocytes.

In essence this is an interesting study that contributes to a better understanding how plasticity between helper and cytotoxic ILCs is regulated. Although such plastic properties have been documented before in mice and human, the transcriptional regulators that drive such a switch are incompletely understood. The quality of the presented work is, however, quite diffuse: some parts of the study are thoroughly and well executed, while other parts fails short in terms of experimental design and use of proper controls. Moreover, the work has mainly a descriptive character that is devoid of any functional (in vivo) experiments to validate their findings and to evaluate the relevance of their findings. Finally, although the authors attempt to generalize their finding to other organs, this is only partly demonstrated: (a) the bifurcation of Hobit and Eomes expression in liver resident ILC1s and NK cells, respectively, is not conserved between other (studied) tissues; (b) tissues in which ILC1s have been demonstrated to have important function were not studied (lung, gut, skin); (c) assessment of SG and kidney ILC1s is limited and is not proportional to hepatic ILC1s. Taken together, it is potentially an interesting study, but their findings and claims need to be further substantiated by experimental proof.

Major comments
Conceptually, I don't understand why IFNg producing "helper" ILC1s are considered immature as compared to "cytotoxic" ILC1s. Both have effector functions and both ILC1 modalities have previously been demonstrated to be instrumental in protecting against specific threats: following MCMV infections DC-derived IL-12 trigger an early non-redundant protective IFNg response by hepatic ILC1s (Weizman et al, Cell 2016), and Il-15 was shown to drive cytotoxicity in ILC1s in the tumor microenvironment (Dadi et al, Cell 2017)). Plasticity between these modalities has also been documented. For example, TGFb was sufficient to convert cytotoxic ILC1s towards TNF and IFNg producing ILC1s (Gao et al. Nat Imm, 2017; Cortez et al., Nat. imm, 2017; Hawke et al, JI, 2020). Thus although HOBIT is instrumental in driving TCF1+ CD127+ towards TCF1-CD127- ILC1s, this seems to be independent of their maturation stage and thus should this concept be reconsidered throughout the manuscript.

Although the authors focus on an important diversification in function of ILC1s that seems to be controlled by HOBIT, no attempts were undertaken to dissect the cues that drive this diversification. As mentioned above, IL-12 IL-15 and TGFb determine the ILC1 state, and thus likely involved in the regulation of Hobit expression. Indeed, it has been reported that Hobit is controlled by T-bet in an Il-15 dependent manner in Trm (MacKay et al. Immunity 2015). The manuscript would largely benefit from such a functional (in vivo) analysis as this is currently lacking. Also, a functional validation of the described mechanism hampers the interpretation and significance of the finding.

Do "cytotoxic ILC1s" following induction of conditional Hobit KO mice revert to CD127+ "helper" ILC1s in vivo?

In homeostasis (deviations from homeostasis were not tested) CD127+ and CD127- ILC1s occur at a similar ratio. However, in culture and in reconstitution experiments, this balance was lost: all cells down-regulated CD127 and acquired the capacity to produce granzymes. Thus the used culturing conditions (what are the "homeostatic cytokines"? What is the role of Notch signaling?) clearly do not reflect the in vivo status quo, and directional statements are therefore premature. Moreover, the authors highlight the proliferative capacity of CD127+ ILC1s as compared to CD127- ILC1s, but this was not shown. To demonstrate the expansion of CD127+ ILC1s and subsequent differentiation in vitro, one could perform a cell tracer violet experiment (see e.g. Bernink et al., Nat. Imm 2013), or more elegant, to infer the directional lineage relationships in vivo, one should perform a lineage tracing experiment. The in vivo reconstitution experiment presented in this manuscript, however, does not add much as those mice were sub-lethally irradiated, lacked lymphocytes, and thus does not reflect steady state situation. Of note, although the rationale for using such animals is not clear to me, the analysis of these mice is fairly limited. As previous research highlighted the plastic properties of ILC subsets, it is reasonable to assume that analysis of other organs (gut, lung, skin, SG, kidney) would help interpreting the data. For example, do CD127+ ILC1s maintain their phenotype in kidney following reconstitution?

The switch of IFNg (and perhaps TNFa)-producing "helper" ILC1s towards granzyme-producing "cytotoxic" ILC1s is only poorly substantiated by experimental proof. Please show stainings for INFg in conjunction with granzymes or provide ELISA-based assays to confirm their switch towards "cytotoxic" ILC1s. Also, proper controls are lacking which are here essential as in vitro culturing assays often result in down-regulation of certain (surface) markers. Thus to validate the presented in vitro data, controls like NK cells and ILC3s need to be included in every experiment to confirm the validity and specificity of ILC1 phenotype.

The performed in vitro experiments (extended figure 2c) show a clear up-regulation of Eomes (>10% in CD127- ILC1s) raising the question whether the used sorting strategy was sufficient to discriminate between NK cells and ILC1s. Throughout the manuscript, do all sorted ILC1s express Hobit and no Eomes? From the presented data in figure 1 it is unclear whether all NK1.1+ CD49a+ CD49b- ILC1s are captured by the HobitWT/Tom reporter. This should be presented. In figure 1d, for example, the pre-gating strategy is not clear and in figure 1e (far right panel) suggest that not all presented ILC1s are Hobit+. Does these cells also include other ILC subsets or NK cells?Do those cells express Eomes? Please clarify for each figure shown the gating strategy.

Previous work of these authors (Lunemann et al., Scient. rep, 2017) identified HOBIT expression in human hepatic NK cells, but whether the described mechanism is also operative in human ILC1s was not investigated in this study. This would be of interest as a recent report described the of "helper" to "cytotoxicity" transition in human ILCs(Krabbbendam et al., EJI 2020).

Minor comments
- Why is the surface receptor CD127 stained intracellularly? Please clarify.

- The observed change in cellular state is not functionally tested. Do cytotoxic ILCs have the capacity to kill? This needs to be demonstrated, for example using K562 cells.

- Extended data 1b (2nd and 3rd panel) axis incorrectly annotated.

- "….defining them as bona-fide ILC1…." Here the figure# should be added.

- Why are MLN-derived ILC2 and ILC3 used as controls and not controls from the same tissue (extended figure 1d)?

- The authors use OP9-DL1 cells for culturing ILC1s. Have the authors used OP9-Neo to control for biased expansion towards "cytotoxic" ILC1s as a consequence of constitute Notch signaling?

Reviewer #2:
Remarks to the Author:
In this article, the authors used various mouse models, single-cell analyses, in vitro culture and adoptive transfer experiments to show the existence of three stages of differentiation along the ILC1 developmental pathway. These three stages include cKit+CD127+Tcf7high, cKit-CD127+Tcf7int and cKit-CD127-Tcf7- cells, all stages expressing NK1.1 and therefore being already committed to the ILC1 lineage. Moreover, they show that the transcription factor Hobit is required for ILC1 differentiation to the TCF7int and TCF7low stages. Altogether, these data are novel and original and this paper is therefore a good candidate for publication in NI. Yet,

Main points

1) In their previous study (Zeis et al, Immunity 2020), the authors identified circulating ILCPs in the mouse lung. Their phenotype appears strikingly similar to that of early-stage ILC1s identified here, with the exception of NK1.1. Thus, it would be useful to determine how TCF7high ILC1s compare to ILCPs in terms of global gene expression patterns. Are there circulating ILCPs in the liver?
2) One possibility could be that ILCPs turn on T-bet expression as soon as they enter the liver (due to local microenvironments) and therefore quickly engage into the ILC1 program. But these cells are arguably still at an early stage of differentiation. This raises the question of their pluripotency. Can they differentiate into other ILC lineage when placed in the appropriate environment?
3) CD127+ CD161+ ILC1s were originally described in the human gut. Have the authors looked at the phenotype of mouse gut ILC1s? Do they only find CD127+ ILC1s as well?

Studying the phenotype of ILC1s in lymphoid organs (lymph nodes, spleen, bone marrow) would be also important.
4) Throughout the paper, the authors refer to CD127+ ILC1s as "helper" and CD127- ILC1s as cytotoxic. I grant that previous studies documented this point, but I think it would be important to confirm it in the present study ie perform cytotoxicity assays and measure the cytokine secretion potential of both ILC1 stages.
5) The present paper is limited to the description of ILC1 differentiation at steady state. I think it would be important to provide at least some data on how this differentiation is impacted in pathological situations (ie inflammation, cancer or infections).

Reviewer #3:
Remarks to the Author:
This study identifies two subsets of hepatic ILC1, which vary with respect to their gene expression. CD127+ ILC1 are less mature and can differentiate and become Gzm+ ILC1. The authors examine mice deficient for the Hobit, a known transcription factor essential for the development of hepatic ILC1. Hobit KO mice fail to generate Gzm+ ILC1 whereas CD127+ ILC1 still develop. Finally, the salivary gland and kidney contain mainly CD127+ immature ILC1, which can be further differentiated in vitro.
The study provides a detailed description of hepatic ILC1 development and the experiments are carried out to a high standard. However, the following points have to be addressed before the manuscript can be considered for publication.

Figure 1:

Did the authors exclude the possibility that CD127+ ILC1 are derived from ILC2 or ILC3?
Is there a function for Gzm in ILC1?
Are Gzm+ ILC1 more cytotoxic towards target cells?
Can the authors demonstrate a non-redundant function for Gzm+ ILC1?

Figure 2:
This could be a Suppl. Figure since these experiments are not central to the main conclusions. Stats are missing.

Figure 3/4: How do the authors exclude that the two subsets of hepatic ILC1 identified have different functional specializations rather than being precursor and effector cells?
Is there a role for IL-2 in promoting Gzm+ ILC1 in vivo?

-
Figure 5:

Which molecules regulate Hobit expression in ILC1?

Are Il18r1, c-kit, Tcf7, and GzmA/B direct target genes of Hobit?

Figure 6: No statistics throughout except the final panels.

Figure 7:
What are the cues regulating Hobit expression in different tissues?

Figure 8:

In which developmental stage is Hobit induced during ILC1 development? Is Hobit expressed in ILC1 or precursors before populating the liver?

Author Rebuttal to Initial comments

Effector differentiation downstream of lineage commitment in ILC1 is driven by Hobit across tissues
(NI-A31400, Friedrich et al.)

Response to the Reviewers

We would like to thank the referees for their constructive criticism and suggestions to further improve our manuscript. We were happy to see that all referees recognize the novelty, originality and impact of our work. As outlined in more detail in the point-by-point response below, we have addressed the reviewer's points through a large set of new experiments and analyses.

We will here summarize our **response to four major points** that have been brought up by all reviewers:

1) Does Hobit act at the ILCP or ILC1 precursor level, or does it mark lineage-committed ILC1?

We have addressed this question using Hobit reporter and Hobit fate-mapper mice, and Hobit KO mice. We also provide a detailed comparison of gene expression patterns of ILCPs with the early Tcf1^{hi} ILC1s that we identified in this manuscript (new Figures 4 d-m, 6 a-d and Extended Data 5 d-g).

Together, our analyses suggest, that Hobit expression specifically marks ILC1 after separation from the other ILC lineages.

2) Functional validation of the effector functions of CD127^{+} Gzm^{lo} versus CD127^{-} Gzm^{hi} ILC1.

This important point is addressed with new cytotoxicity, cytokine production and proliferation assays that validate the suggested effector functions and proliferative potential of CD127^{+} Gzm^{-} versus CD127^{-} Gzm^{+} ILC1s (new Figures 1g-h, Extended Data 1f and 3f and Figure R6). Our new analyses confirm that the transition of CD127^{+} into CD127^{-} ILC1 is accompanied by the downregulation of proliferative potential and the upregulation of the ability to kill target cells, while the capacity for cytokine production is retained.

3) Generalizability of our findings across tissues.

The applicability of our original findings for ILC1 in other tissues than the liver was addressed further using in depth analysis of ILC1 in various tissues, including the liver, kidney, salivary gland, small intestine and LN. Our new analyses include in vitro differentiation assays, Hobit reporter and KO mice, mixed bone marrow chimeras, and Hobit-floxed mice (new Figures 7a-c, 8d-g and Extended Data 6j-q, 7a-j and 8f).

These results clearly support our previous conclusion that ILC1 across tissues are connected through a uniform developmental path, and share the potential to differentiate toward CD127^{-} Tcf1^{lo} Gzmab^{hi} effector cells. As we show, Hobit is expressed in ILC1 in all analyzed tissues and regulates the expression of Tcf1, Il18r1 and granzymes in this lineage in all analyzed tissues. While in the steady state not all effector states are realized in every tissue (e.g. kidney, LNs and small intestine), we now show that this differentiation path is initiated *in vivo* in these tissues during inflammation, as evidenced by the downregulation of Tcf1 and the concomitant increase in granzyme expression (see below, and new Figures 8d-g and Extended Data 8f).

4) Is the proposed effector differentiation occurring *in vivo* during inflammation?

To address this point, we have analyzed Foxp3^{DTR} mice that have allowed us to create an inflammatory environment *in vivo* upon elimination of Foxp3^{+} regulatory T cells. Interestingly, we found that depletion of regulatory T cells induced the *in vivo* differentiation of CD127^{hi} Tcf1^{hi} Gzm^{-} ILC1s toward CD127^{-} Tcf1^{lo} Gzm^{+} cells in the kidney, gut and LNs (new Figure 8g and Extended Data 8f).

Together, these experiments delineate the developmental stage at which Hobit expression is initiated and confirm that this transcription factor marks lineage commitment of ILC1 across tissues. Our analyses provide strong evidence for a conserved developmental trajectory of ILC1s across tissues, suggesting that committed ILC1 emerge as Tcf7^{hi} Hobit^{+} cells that diversify into distinct effector populations that segregate by proliferative potential and expression of effector molecules.
The analyses of Treg cell depleted mice indicates that this differentiation potential is actively regulated *in vivo* in the respective tissue-niches and by inflammatory stimulation. Together, our findings establish a novel conceptual framework that connects phenotypically diverse ILC1 along a uniform differentiation pathway driven by Hobit.

We have included additional analyses and have addressed all suggestions and concerns of the reviewers in our point-by-point response below. In the revised manuscript we have marked all changes in blue, and have highlighted all new figures in red (also in this letter).

We thank the reviewers for the constructive discussion and we believe that addressing their points has further improved the significance of the manuscript. We hope that you will find our manuscript of sufficient interest and quality for publication in *Nature Immunology*.

Kind regards,

Georg Gasteiger
(on behalf of Christin Friedrich, Renske Taggenbrok, Klaas van Gisbergen, and co-authors)

Point-by-point response:

Reviewer #1

In this study Gasteiger and colleagues investigated the putative heterogeneity of liver-resident ILC1s. The authors demonstrate that previously described hepatic IFNg-producing "helper" ILC1s and granzyme and perforin producing "cytotoxic" ILC1s are in fact the same cell type that can present itself in distinct modalities. This study builds on previous work that identified Hobit as an essential regulator of hepatic ILC1 tissue-residency and survival in mice. Here, employing flow cytometric and RNA sequencing techniques the authors demonstrate that the cellular state is determined by the transcriptional regulator Hobit, which pushes "helper" ILC1s towards "cytotoxic" ILC1s. Differentiation towards cytotoxic ILC1s was accompanied by the gradually loss of Kit, Tcf7 and Cd127 expression and (potentially as a consequence of that) loses its potential to proliferate as compared to helper ILC1s. Finally the authors demonstrate that the Hobit-dependent cytotoxicity is not a hepatic ILC1-specific phenomenon, but seems to be conserved in salivary glands and kidney resident innate lymphocytes.

In essence this is an interesting study that contributes to a better understanding how plasticity between helper and cytotoxic ILCs is regulated. Although such plastic properties have been documented before in mice and human, the transcriptional regulators that drive such a switch are incompletely understood.

We thank the reviewer for the positive assessment of our work and for pointing out the novelty and significance of our study. We would like to emphasize, however, that we have not investigated plasticity of ILCs (a term that is often used for e.g. ILC3s acquiring functions of ILC1s, or ILC2s acquiring functions of ILC3s, etc), but rather propose a novel mechanism for the effector differentiation of lineage-committed ILC1s driven by Hobit across tissues. In fact, our newly added data employing Hobit fate-mapping, as detailed in our response to Reviewer 2, shows that the identified subsets are fully committed to the ILC1 lineage (new Figure 4 h-m).

The quality of the presented work is, however, quite diffuse: some parts of the study are thoroughly and well executed, while other parts fails short in terms of experimental design and use of proper controls. Moreover, the work has mainly a descriptive character that is devoid of any functional (in vivo) experiments to validate their findings and to evaluate the relevance of their findings.

We politely disagree with the reviewer. Our studies reveal sequential mechanisms of ILC1 lineage commitment and effector maturation that are conserved across tissues. Our analyses demonstrate that ILC1 emerge as Tcf1^{hi} cells in the periphery and acquire a spectrum of effector phenotypes through a uniform Hobit-dependent differentiation pathway. Our conclusions are based on:

- scRNA-Seq analysis of WT and Hobit^{KO} ILCs, revealing previously undescribed, distinct subtypes of ILC1
- computational inference of a differentiation trajectory
- experimental validation of this trajectory *in vitro* and *in vivo*, revealing the unique potential for expansion and effector differentiation of Tcf1^{hi}, but not Tcf1^{lo}, ILC1
- genetic studies, demonstrating that Hobit is a key transcription factor driving ILC1 effector differentiation across tissues
- use of Hobit reporter and KO mice to demonstrate that Hobit has a similar function in Eomes^{+} tissue-resident NK cells in salivary glands
- conditional deletion of Hobit *in vivo* (new mouse model) demonstrating the continuous requirement for Hobit to maintain a diverse spectrum of effector states of ILC1, including Tcf1^{lo} cytotoxic ILC1
- a large set of new data, including Hobit fate-mapping and functional validation (cytotoxicity, cytokine production, *in vivo* differentiation)

Our manuscript reports novel mechanistic insights into the developmental relationship of committed ILC1, as well as the gene expression programs enabling their effector differentiation. We would like to emphasize that ILCs (as other innate lymphocytes) undergo developmental effector differentiation by "default" during homeostasis (in contrast to adaptive T cells, which are maintained as naïve cells and require priming in response to e.g. infection). This notion is further supported by the detection of all

effector states in germfree mice (Extended Data Figure 2f). We therefore think that it is highly relevant to investigate the development of ILC1 under homeostasis.

Nevertheless, to address the reviewer's concern, we have expanded our analysis using *in vivo* depletion of Foxp3^{+} Treg cells to trigger tissue inflammation and local activation of tissue-resident ILCs(Gasteiger et al., 2015) These findings show that ILC1 undergo effector differentiation in response to inflammation (new Figure 8g and Extended Data 8f). We have detailed our data using this experimental system of tissue inflammation further below, and in response to reviewer 2.

Finally, although the authors attempt to generalize their finding to other organs, this is only partly demonstrated:
(a) the bifurcation of Hobit and Eomes expression in liver resident ILC1s and NK cells, respectively, is not conserved between other (studied) tissues;

We would like to point out that we do not investigate the bifurcation of Eomes^{+} NK cells and ILC1. The main focus of our manuscript is the differentiation pathway of Hobit^{+} Eomes^{-} ILC1 that have already committed to this lineage. We identify Hobit as a factor driving final differentiation steps of already committed ILC1s across tissues. In support of this view, our analysis of the ILC1 compartment in livers, kidneys and LNs of NKp46^{Cre} x Eomes$^{fl/fl}$ mice (Extended Data Figure 2b and Figure R1) support the idea, that the ILC1 subtypes that we identify develop independently of Eomes, and represent bona-fide Eomes^{-} ILC1.

We would also like to highlight that Hobit expression is not restricted to Eomes^{-} ILC1. In fact, we have previously reported that Hobit has functions in NKT cells, and tissue-resident T cells as well (Mackay et al., 2016; van Gisbergen et al., 2012; Zundler et al., 2019). Here, in addition to our analyses of Eomes^{-} ILC1, we report that Eomes^{+} tissue-resident NK cells also express Hobit (Extended Data 8a). Intriguingly, we found that these cells also segregate into Tcf1^{hi} Grzm^{-} and Tcf1^{lo} Grzm^{+} subsets, and that Hobit regulates the effector differentiation of these cells in a very similar manner as in ILC1s. These analyses suggest that Hobit may regulate a conserved cascade of effector differentiation in Eomes^{-} as well as Eomes^{+} tissue-resident group 1 ILCs. These findings are particularly interesting in light of the reported expression of Hobit in tissue-resident and other NK cells in humans (see below). While we are very excited about these findings, we would like to stress that they do not address mechanisms of the bifurcation of Eomes^{+} NK cells and ILC1, which has not been the focus of our work.

(b) tissues in which ILC1s have been demonstrated to have important function were not studied (lung, gut, skin);

We have included analysis of additional tissues to expand the scope of our study as suggested by the reviewer. Distinct from human skin, ILC1s are extremely rare cell types in steady state murine skin, at least under the conditions of our SPF facilities. In the lung, a large fraction of Eomes- cells represent circulating cells that appear to be located in the vasculature and do not fit the definition of tissue-resident group 1 ILCs that we investigate in this study(Gasteiger et al., 2015). We have therefore included extensive new data on small intestine and mesenteric lymph node ILC1 (new Figures 7a-c, 8d-g and Extended Data 6j-q, 7e-f and 8f) which support the key findings and claims on liver, kidney and salivary gland ILC1 in the original manuscript.

(c) assessment of SG and kidney ILC1s is limited and is not proportional to hepatic ILC1s.

We have performed scRNA-Seq of hepatic WT and Hobit deficient ILC1s to identify Hobit-dependent genes and differentiation patterns. From there, we have expanded our analysis to different tissues. We now include several additional analyses characterizing ILC1 across tissues:

- we have performed novel analyses of Nkp46^{Cre} x Eomes$^{fl/fl}$ mice confirming that the investigated ILC1 subsets are Eomes-independent and distinct from Eomes^{+} cNK (Fig. R1). Due to the presence of ICL1-like Eomes^{+} cells in the SG, which cannot be distinguished from Eomes^{-} ILC1 after depletion of Eomes, we have not included these analyses.
- We have validated ILC1 subsets and major Hobit-dependent gene expression patterns in the SG, kidney, small intestine and LN in WT versus Hobit KO mice (new Figure 7b)

- we have studied the differentiation potential of ILC1 from kidney and small intestine *in vitro* (new Figure 8d-f), and upon Treg cell depletion *in vivo* (new Figure 8g, Extended Data 8f).
- we have performed the conditional deletion of Hobit in $Ubi^{CreERT2}$ x $Hobit^{fl/fl}$ mice and analyzed diverse tissues (Extended Data 7g-j)
- we have included analyses of mixed bone marrow chimeras that confirm the cell intrinsic requirement for Hobit in all analyzed tissues (new Extended Data 7a-d)

We feel that the revised manuscript contains a proportional analysis of ILC1 subsets across several tissues in which ILC1 have reported biological functions.

Taken together, it is potentially an interesting study, but their findings and claims need to be further substantiated by experimental proof.

Major comments
Conceptually, I don't understand why IFNg producing "helper" ILC1s are considered immature as compared to "cytotoxic" ILC1s. Both have effector functions and both ILC1 modalities have previously been demonstrated to be instrumental in protecting against specific threats: following MCMV infections DC-derived IL-12 trigger an early non-redundant protective IFNg response by hepatic ILC1s (Weizman et al, Cell 2016), and Il-15 was shown to drive cytotoxicity in ILC1s in the tumor microenvironment (Dadi et al, Cell 2017)).

We thank the reviewer for pointing out that this was apparently not sufficiently clear in the initial manuscript: we do not consider helper ILC1 as immature, but as competent effector cells that are upstream of cytotoxic ILC1 in a developmental path. We have shown in the original manuscript that the $CD127^+$ (and even $cKit^+$) helper ILC1 make IFNγ (Figure 1g and 4b), and our scRNA-Seq data reveals expression of other effector molecules in these cells (Figure 2a). We also stated in the results section:

"$cKit^+$ $Tcf7^{hi}$ ILC1 expressed NK1.1, NKp46, Tbet and Hobit and produced IFNγ upon stimulation, suggesting that our analysis identified early but committed differentiation stages of effector ILC1s (Fig. 4g, h and data not shown)"

In the revised manuscript, we highlight the effector function of $CD127^+$ ILC1 in several instances, and support it with new data:

"Indeed, while $CD127^+$ had a modestly increased capacity to produce the cytokines IFNγ, TNFα and GM-CSF, $CD127^-$ ILC1 were able to kill YAC target cells more efficiently than their $CD127^+$ counterparts (Fig. 1g, h and Extended Data Fig. 1f)."

"*Reminiscent of the potential for cytokine production and cytotoxicity (Fig.1g, h), expression of these genes was not mutually exclusive among $CD127^+$ and $CD127^-$ subsets but instead appeared to be gradually increased in one or the other cluster, raising the possibility that these ILC1 subsets represented extremes of a continuous spectrum of effector differentiation states."*

We have included a new Figure 6 entitled "Hobit is specifically expressed in committed ILC1" that delineates $Tcf1^{hi}$ early ILC1s from immature ILCPs, and we have revised the manuscript to avoid descriptions like "more immature", etc., which can be misleading in this regard.

We have also highlighted this in the revised discussion:

"Importantly, $Tcf1^{hi}$ early ILC1 already have the ability to produce IFNγ and other pro-inflammatory mediators, suggesting that they represent a mature ILC1 subset that can exert effector functions. Downstream differentiation stages appear to retain the potential to release pro-inflammatory cytokines, but gradually acquire other effector functions such as the production of Fgl2, granzymes and the ability to efficiently kill target cells."

Plasticity between these modalities has also been documented. For example, TGFb was sufficient to convert cytotoxic ILC1s towards TNF and IFNg producing ILC1s (Gao et al. Nat Imm, 2017; Cortez et al., Nat. imm, 2017; Hawke et al, JI, 2020).

The reviewer refers to papers that describe the conversion of Eomes^{+} NK cells into Eomes^{-} ILC1. Cortez *et al* demonstrated, that this conversion depends on TGFb(Cortez et al., 2017) however the same authors demonstrated that the phenotype of "bona-fide" Eomes^{-} ILC1s, e.g. in the liver, is independent of TGFβ(Cortez et al., 2016). In our manuscript, we do not investigate plasticity from NK cells towards ILC1 (see also above). Instead we identify a 3-stage developmental program of Eomes^{-} independent ILC1s that is driven by the transcription factor Hobit.

Thus although HOBIT is instrumental in driving TCF1+ CD127+ towards TCF1-CD127- ILC1s, this seems to be independent of their maturation stage and thus should this concept be reconsidered throughout the manuscript.

We thank the reviewer for acknowledging the instrumental function of Hobit in driving ILC1 effector differentiation. We also agree with the reviewer that Hobit acts throughout the entire differentiation process to drive effector diversification within committed ILC1. In the revised version of the manuscript we highlight expression changes of putative direct Hobit targets along the trajectory (new Figure 6e, f). Moreover, as detailed above, we have emphasized that both Tcf1^{+}CD127^{+} and Tcf1^{-}CD127^{-} ILC1 are mature cells with regard to, for example, their ability to produce IFNγ (new Figure 1g and R6). However, we have observed "functional maturation" between Tcf1^{+}CD127^{+} and Tcf1^{-}CD127^{-} ILC1 in terms of downregulation of proliferation potential (Figure 2c and new Extended Data 3f) and upregulation of cytotoxic molecules including Gzma, Gzmb and GzmC (Figure 1f, 2b, e and Extended Data 3c, h). Thus, we conclude that Tcf1^{+}CD127^{+} are already mature cells that can produce IFNγ, but that require transition into Tcf1^{-}CD127^{-} cells to acquire robust cytotoxic function. We have provided a discussion of this differentiation as reminiscent of T cell subset diversification, and we have carefully rephrased the revised manuscript to avoid terms like "immature" etc.

Although the authors focus on an important diversification in function of ILC1s that seems to be controlled by HOBIT, no attempts were undertaken to dissect the cues that drive this diversification. As mentioned above, IL-12 IL-15 and TGFb determine the ILC1 state, and thus likely involved in the regulation of Hobit expression. Indeed, it has been reported that Hobit is controlled by T-bet in an Il-15 dependent manner in Trm (MacKay et al. Immunity 2015). The manuscript would largely benefit from such a functional (in vivo) analysis as this is currently lacking. Also, a functional validation of the described mechanism hampers the interpretation and significance of the finding.

As the reviewer highlights, our work reveals an important Hobit-dependent mechanism of ILC1 effector diversification. These findings are functionally validated through a broad range of analyses using constitutive and conditional deletion of Hobit. While not the direct focus of our work, we agree that unraveling the instructive cues of Hobit regulation may further improve our understanding of the final differentiation steps of ILC1. Therefore, we have performed *in vivo* neutralization experiments of Il15 to analyze its influence on ILC1 effector diversification, as suggested by the reviewer (R4). Furthermore, we have analyzed mice deficient in STAT4 (an essential signaling molecule for Il12), mice deficient in IL18r1 and mice deficient in IL2 signaling (NKp46^{Cre} x CD25$^{fl/fl}$ mice) for effector differentiation of ILC1 (R2, R3 and R5). Our analyses therefore indicate that, under steady state conditions, Il12, Il18, Il2 and Il15 (beyond the essential function if Il15 during ILC1 development (Daussy et al., 2014; Klose et al., 2014) are not required to regulate the differentiation of ILC1 into the effector subtypes that we have identified in this study. As we show that this differentiation requires Hobit (Figure 5), we conclude that these cytokines are not required to induce Hobit expression.

Conversely, Cortez *et al.* have previously shown that ILC1s in the liver develop independently of TGFβ to normal numbers and have unaltered expression of CD49a, CD69 and CD73 in Ncr1-Cre x Tgfbr2-floxed mice(Cortez et al., 2016) (Cortez et al., Immunity 2016 Fig S1). As we found that numbers of ILC1s in the liver as well as expression of these genes are significantly reduced in Hobit KO mice, we conclude that TGFβ is not required to drive Hobit expression.

In an additional attempt to address the reviewers request, we have additionally sorted BM ILCPs and performed established *in vitro* differentiation assays that we have previously established in the lab(Zeis et al., 2020) () to determine whether cytokines Il7, Il2, Il15, Il12, Il18 and TGFβ, or combinations thereof,

are able to induce Hobit expression. We have succeeded in differentiating CD49a^{+} Tbet^{+} Eomes^{-} ILC1-like cells, however, these cells did not upregulate Hobit as assessed with the Hobit-reporter and fate-mapper allele (data not shown).

Our data therefore indicate that additional factors, likely from within the respective tissue niches, are required to instruct Hobit expression. We feel that the main focus of our manuscript is the function of Hobit in driving ILC1 effector differentiation, and that unraveling the instructive cues of Hobit will require future work that goes beyond the current focus of the manuscript. As we are already reaching the limit of the main manuscript as well as the Extended Figure format, we propose to not include these analyses. Nevertheless, we have included a section in the discussion to account for the reviewer's suggestion in the manuscript:

"The instructive signals driving the differentiation of committed ILC1 in vivo remain unclear. Our in vitro cultures suggest that Il2 and Il15 can contribute to the transition of helper-like ILC1 into cytotoxic-like ILC1. Il2 may be more relevant for ILC1 differentiation under inflammatory conditions as occurs upon depletion of regulatory T cells, while Il15 may dominate under homeostatic conditions, as supported by its essential contribution to ILC1 development(Daussy et al., 2014; Klose et al., 2014). In preliminary analyses, the phenotype of liver ILC1s was not altered in STAT4-KO and Il18r1-KO mice, or when we neutralized Il15 (data not shown). Similarly, hepatic ILC1s had no detectable change in phenotype when lacking Tgfbr2(Cortez et al., 2016). These observations suggest that these cytokines do not directly maintain or induce Hobit and highlight that additional work will be required to address the instructive signals of Hobit expression and the differentiation of lineage-committed ILC1."

As detailed in our response to reviewer 2, we have included new data on ILC1 in Foxp3^{DTR} mice that enable deletion of regulatory T cells, thereby creating an inflammatory setting. We have previously demonstrated that ILC1 respond locally, as tissue-resident cells in that experimental model(Gasteiger et al., 2015). Now, we observed that ILC1s that do not express granzymes at steady state (such as in the kidney, small intestine and lymph nodes), undergo effector differentiation and diversification upon Treg cell depletion, as evidenced by the downregulation of Tcf1 and the concomitant increase in granzyme expression (new Fig. 8g and Extended Data Fig. 8f).

These findings confirm the *in vivo* differentiation potential suggested by our previous analyses and are consistent with the idea that ILC1s are actively retained in their Tcf1^{hi} Gzm^{-} state in some tissues during homeostasis, but can gain additional effector functions in the absence of Treg control, or as a consequence of strong inflammation. We believe that these data provide a very exciting *in vivo* validation of the concepts emerging from our study.

Do "cytotoxic ILC1s" following induction of conditional Hobit KO mice revert to CD127+ "helper" ILC1s in vivo?

We think that the question of the reviewer is interesting, but addressing this question would require models to fate-map the specific ILC1 differentiation stages that we have identified here. To the best of our knowledge, such models are currently not available (see also below).

Using the novel Hobit-floxed mice that we have generated, we have observed that conditional deletion of Hobit induces a pronounced increase in Tcf1 and Il18r1 expression that is accompanied with a reduction of granzyme-expressing cells (Figure 6g-i and New Extended Data 7g-j). These findings are consistent with all the other presented experiments suggesting that a lack of Hobit blocks the transition of Tcf1^{hi} early ILCs towards downstream Gzm^{+} effector cells. However, the experimental setup does not allow us to formally distinguish between the reversion of cytotoxic ILC1 to helper ILC1 after conditional Hobit ablation or, alternatively, the accumulation of helper ILC1, caused by a blockade to differentiate into ILC1 after Hobit ablation. We have added these in the revised manuscript to address the reviewer's point:

"Although this analysis cannot formally distinguish between the reversion of CD127^{-} Tcf1^{lo} Gzm^{hi} ILC1 to CD127^{+} Tcf1^{hi} Gzm^{lo} ILC1 after conditional Hobit ablation versus the accumulation of CD127^{-} Tcf1^{hi} Gzm^{lo} ILC1, caused by a blockade to differentiate into CD127^{-} Tcf1^{lo} Gzm^{hi} ILC1 after Hobit ablation, these data suggest that hepatic ILC1 continuously require Hobit expression to regulate the balanced differentiation between Tcf1^{hi} Gzm^{lo} vs Tcf1^{lo} Gzm^{hi} effector ILC1s."

In homeostasis (deviations from homeostasis were not tested) CD127+ and CD127- ILC1s occur at a similar ratio. However, in culture and in reconstitution experiments, this balance was lost: all cells down-regulated CD127 and acquired the capacity to produce granzymes. Thus the used culturing conditions (what are the "homeostatic cytokines"? What is the role of Notch signaling?) clearly do not reflect the in vivo status quo, and directional statements are therefore premature.

Given the multitude of signals that ILCs perceive in their tissue niches, we think that it may be difficult to model a "homeostatic" situation *in vitro*. However, to address the reviewer's question to the best of our ability, we have performed additional culture experiments with potential "homeostatic cytokines" including Il7 and Il15. We have also explored the impact of the Notch pathway in our culture system. Consistent with previous reports that Il7 is dispensable for the maintenance of ILC1 *in vivo(Daussy et al., 2014; Klose et al., 2014)* we observed that Il7 could not maintain CD127^{+} or CD127^{-} ILC1 when cultured in the presence of Il7 alone, or in OP9 or OP9-DL1 cultures supplemented with Il7, despite their expression of CD127/IL7Ra (data not shown). In contrast, both ILC1 subsets could be maintained on Il15, reflecting the *in vivo* status, as anticipated by the reviewer. We have included these new data in the new Extended Data 3a-e.

"Because Il2 may activate ILC1, we tested their differentiation in the presence of Il15, which is an essential homeostatic cytokine and differentiation factor for ILC1(Daussy et al., 2014; Klose et al., 2014). The overall expansion was significantly reduced in the Il15 versus Il2 supplemented cultures, yet CD127^{+} ILC1 still gave rise to Gzma and Gzmb-expressing CD127^{-} cells. Notably, a higher fraction of cells retained their original phenotype (CD127^{+} Gzma^{-} Gzmb^{-} in the presence of Il15, consistent with the idea that Il15 maintains a proportion of ILC1 in an early differentiation state, and reminiscent of homeostatic conditions in vivo (Extended Data Fig 3e). In contrast to CD127^{+} ILC1, CD127^{-} ILC1 showed an impaired expansion in all experimental conditions that we tested, and remained CD127^{-} while further increasing CD3γ, Gzma and Gzmb expression during culture (Fig. 2c-e and Extended Data Fig 3a-e)."

We have also included new data addressing the putative role of notch in these cultures, as requested by the reviewer:
"This differentiation was independent of stimulation with the notch ligand DL1, as similar results were obtained with OP9 and OP9-DL4 feeder cells (Extended Data Fig 3d)."

The reviewer further questions statements about the directionality of the developmental trajectory of ILC1. We have addressed this point further below.

Moreover, the authors highlight the proliferative capacity of CD127+ ILC1s as compared to CD127- ILC1s, but this was not shown. To demonstrate the expansion of CD127+ ILC1s and subsequent differentiation in vitro, one could perform a cell tracer violet experiment (see e.g. Bernink et al., Nat. Imm 2013), …

We believe that the reviewer might have overlooked the Figures 2c and 3f in which we show that CD127^{+} cells expand about 6x more than CD127^{-} cells and that cKit^{+} CD127^{+} cells expand about 20x more than CD127^{-} cells. This dramatically increased expansion potential likely involves increased proliferative capacity. Indeed, cell tracer violet experiments performed in response to the reviewer's suggestion show that CD127^{+} ILC1 have increased proliferative potential compared to CD127^{-} ILC1 (new Extended Data 3f) in line with the original data regarding expansion of these ILC1 subsets *in vitro* and *in vivo* (Fig. 2c, f and 3f.

… or more elegant, to infer the directional lineage relationships in vivo, one should perform a lineage tracing experiment. The in vivo reconstitution experiment presented in this manuscript, however, does not add much as those mice were sub-lethally irradiated, lacked lymphocytes, and thus does not reflect steady state situation. Of note, although the rationale for using such animals is not clear to me, the analysis of these mice is fairly limited.

The reviewer requests a more thorough *in vivo* assessment of the directionality of the developmental trajectory that we have identified. Such *in vivo* assessment would require an inducible fate-map model driven by a gene that is expressed only at a specific developmental stage of the newly discovered ILC1 subsets, but not expressed by upstream progenitors and downstream ILC1s. To the best of our knowledge, such models are currently not available. The difficulty of targeting a specific developmental stage of ILCs is also highlighted by the fact that until today (and despite strong efforts by the leading labs in the field) there is not even a Cre-model that enables the specific targeting and fate-mapping of ILCs (without targeting progenitors, or T cells and NK cells), independently of their developmental stage.

Due to the lack of such stage-specific fate-mapping models, previous studies have built on three experimental pillars to establish progenitor-product relationships and the differentiation potential of distinct cell populations during hematopoiesis, including the differentiation of ILCPs and ILCs: 1) the phenotypic description of distinct progenitor or ILC subsets including unbiased gene expression analyses, 2) the demonstration of their *in vitro* differentiation potential by culture, and 3) the assessment of their *in vivo* differentiation potential by transplantation into lymphopenic mice (e.g. (Amann-Zalcenstein et al., 2020; Bernink et al., 2015; Ferreira et al., 2021; Harly et al., 2019; Klose et al., 2014; Xu et al., 2019; Yang et al., 2015)).

The characterization of ILC differentiation pathways through these approaches has been extremely influential for future studies although none of these reports could directly test the *in vivo* directionality and relative contribution during homeostasis through stage-specific fate-mapping.

Our work also engages these three experimental "pillars". We use a combination of scRNA-Seq, genetic models and bioinformatical modelling to derive Hobit-dependent differentiation trajectories and to reveal stage-specific, Hobit-dependent gene expression changes. Consistent with the findings from our *in vitro* and *in vivo* validation experiments (see Fig 2c-g, 3f), these analyses suggest that the identified cKit^{+} Tcf1^{hi} "early" helper ILC1 differentiate into Tcf1^{lo} cytotoxic ILC1 through a Hobit-dependent pathway.

We feel that the employed experimental approaches are well established and accepted, and represent the most thorough experimental approaches that are currently available in the field. We would like to further emphasize that we take advantage of new genetic models that have not been reported in the ILC field or have been generated for this study (Hobit-reporter mice, Hobit-conditional KO mice, and now, for the revision: Hobit-Cre mice for fate-mapping). We think that these novel approaches already represent a major step forward.

As previous research highlighted the plastic properties of ILC subsets, it is reasonable to assume that analysis of other organs (gut, lung, skin, SG, kidney) would help interpreting the data. For example, do CD127+ ILC1s maintain their phenotype in kidney following reconstitution?

We have followed the reviewer's suggestion and analyzed ILC1s from different tissues, notably the gut and lymphoid organs (see also above). However, the scarcity of ILC1 in kidney compared to the liver have not allowed us to follow the fate of adoptively transferred CD127^{+} ILC1 in kidney or other organs besides the liver. To directly address the reviewer's question about the developmental plasticity and differentiation potential of ILC1 in different tissues *in vivo*, however, we have generated new Hobit fate-mapping analyses that highlight the stability of the ILC1 lineage during homeostasis, and we have included new data on Treg depleted mice that highlights the differentiation potential of ILC1s in the kidney, mLN and the gut upon inflammation (new figures 4 h-m, 8g, and Extended Data 8f).

The switch of IFNg (and perhaps TNFa)-producing "helper" ILC1s towards granzyme-producing "cytotoxic" ILC1s is only poorly substantiated by experimental proof. Please show stainings for INFg in conjunction with granzymes or provide ELISA-based assays to confirm their switch towards "cytotoxic" ILC1s.

We provided the requested co-labeling experiments for granzymes and IFNγ (Figure R6). We would like to emphasize that we consider cytokine production a central effector function of all ILC1s that is not lost upon the gradual differentiation towards more cytotoxic cells. To make this clearer, we have included new data showing IFNγ, TNFα and GM-CSF production by both CD127^{+} and CD127^{-} ILC1 (new Figure

1g). We have also included analysis of the expression of Gzmbc, in addition to Gzma and Gzmb, in Figure 1f, showing that CD127^{+} cells can express these molecules to some extent (as also stated in our initial submission). Consistent with the gradual changes observed in gene expression (Figure 2a and 3c), these data suggest that CD127^{+} ILC1 differentiate towards CD127^{-} ILC1 by gradually acquiring additional effector functions while forfeiting their expansion potential, reminiscent of the effector differentiation of cytotoxic T cells.

Also, proper controls are lacking which are here essential as in vitro culturing assays often result in down-regulation of certain (surface) markers.

We now show isotype controls for CD127, Il18r1 and Gzmb that we used in our culture assays to confirm our antibody stainings (new Extended Figure 3i). Importantly, we have performed our CD127 staining in these experiments intracellularly, because we are aware that this receptor may be downregulated during culture. We have added this information to the Methods section.

Thus to validate the presented in vitro data, controls like NK cells and ILC3s need to be included in every experiment to confirm the validity and specificity of ILC1 phenotype.

We have performed parallel culture experiments with NK cells and/or ILC3 for head to head comparison with ILC1 in our culture experiments. Given that NK cells but not ILC3 are prevalent in liver, we had included NK cells as controls in all culture experiments using liver ILC1 (new Extended Data 3c). ILC3 rather than NK cells are prevalent in small intestine, and therefore, we have employed ILC3 as controls for culture experiments with ILC1 isolated from this tissue:

The predominant fraction of cultured NK cells maintained Eomes expression and did not express CD3γ (new Extended Data Figure 3c). Intestinal ILC3s did not significantly expand in our cultures and did not gain granzyme expression, potentially due to the presence of notch ligands (data not shown). These findings validate that our analyses reveal the differentiation potential of bona-fide ILC1s.

The performed *in vitro* experiments (extended figure 2c) show a clear up-regulation of Eomes (>10% in CD127- ILC1s) raising the question whether the used sorting strategy was sufficient to discriminate between NK cells and ILC1s.

We have added our post-sort analyses (Extended Data 3b) that show excellent purity of ILC1 subset preparations before culture. We have not reproducibly seen Eomes upregulation in our cultures (see e.g. Extended Data 3c), and have not observed it upon *in vivo* transfer (see Extended Data S3g). The expression of Eomes in ILC1 cultures is very limited compared to that of NK cells cultured under identical conditions (please compare Extended Data Figures 3b and g). Most importantly, Gzmab^{+} cells emerging from ILC1 (and especially CD127^{-} ILC1) show characteristic expression of CD3γ (70-90% in the CD127- ILC1 subset), while only a small fraction of NK cells does express CD3γ. Together with our analyses of Eomes-floxed mice, and the abscence of Eomes^{+} cells in the majority of our ILC1 cultures, these data suggest that CD127^{-} Gzmab^{+} cells emerge from bona-fide ILC1.

Throughout the manuscript, do all sorted ILC1s express Hobit and no Eomes? From the presented data in figure 1 it is unclear whether all NK1.1+ CD49a+ CD49b- ILC1s are captured by the Hobit WT/Tom reporter. This should be presented. In figure 1d, for example, the pre-gating strategy is not clear and in figure 1e (far right panel) suggest that not all presented ILC1s are Hobit+. Does these cells also include other ILC subsets or NK cells? Do those cells express Eomes? Please clarify for each figure shown the gating strategy.

The reviewer correctly points out that a small fraction of ILC1 is not tdTomato positive in this reporter strain. To show this in an unbiased manner, we had used commonly accepted definitions of murine cNK cells as Lin^{-} NK1.1^{+} CD49b^{+} Eomes^{+} cells, and murine ILC1 as (Lin^{-}) NK1.1^{+} CD49b^{-}, Eomes^{-} and NK1.1^{+} CD49a^{+} CD49b^{-} in the initial Figure 1.

Figure 1b shows the live reporter versus CD49b as well as the stained tdTomato protein versus Eomes (the fixation required to stain for Eomes destroyed tdTomato and required antibody staining of the reporter). Figure 1e shows the live reporter in Lin^- $NK1.1^+$ $CD49a^+$ $CD49b^-$ cells. As now highlighted in the figure legend, the pregating in Figure 1d is $CD45^+Lin^-NK1.1^+CD49a^+Eomes^-$.

We have now included a new quantification of Hobit expression in ILC1 subsets versus cNK cells and other ILCs (new Figure 4). These findings show that ILC1, in contrast to $Eomes^+$ cNK cells, express high levels of Hobit. We cannot rule out whether the few $tdTomato^-$ cells represent truly Hobit negative cells, or rather reflect a minor underreporting as observed with many genetic reporter strains. For several analyses, e.g. the sorts for *in vitro* cultures of liver and kidney ILCs, we have therefore followed the commonly accepted definition of murine ILC1 as Lin^- $NK1.1^+$ $CD49a^+$ $CD49b^-$ cells, which make our data comparable to previous and future publications that do not rely on the use of the Hobit-reporter allele.

We would like to mention, that the fixation that is required to stain TdTomato after permeabilization does not combine with reliable transcription factor staining in our hands. Therefore, we cannot exclude the possibility that "live" analyses of the reporter can contain some TdTomato- cells that are "contaminations" of non-ILC1. This appears relevant for ILC3 in the intestine, and therefore we have highlighted this specifically in the revised manuscript (Extended Figure 6a). Importantly, when we sorted Hobit+ ILCs from the intestine and then fixed and stained them they were all RORγt, further confirming a very high specificity of the reporter. In addition, as highlighted above, intestinal ILC3s did not significantly expand in our cultures and did not gain granzyme expression, confirming that these cells are not confounding the conclusions of our analyses.

Most importantly, our new fate-mapping analyses suggest that Hobit expression identifies lineage-committed cells that do not give rise to other ILC subtypes or conventional NK cells (new Figure 4). We have discussed the specific case of $Eomes^+$ tissue-resident group 1 ILC1s in the salivary gland further above. We have clarified gating strategies for all relevant figures in the revised manuscript, as requested.

Previous work of these authors (Lunemann et al., Scient. rep, 2017) identified HOBIT expression in human hepatic NK cells, but whether the described mechanism is also operative in human ILC1s was not investigated in this study. This would be of interest as a recent report described the of “helper” to “cytotoxicity” transition in human ILCs (Krabbbendam et al., EJI 2020).

We have focused our work on the differentiation of ILC1 in mice and we think it will require a separate study with specific focus on human ILC1 differentiation to properly address whether the Hobit-dependent differentiation pathway similarly operates in humans. We consider this work beyond the scope of the present study. We have revised our discussion to highlight this important future question, as well as the broader expression pattern of Hobit in humans (see below).

We thank the reviewer for highlighting that the report of Krabbendam *et al.*, which mainly focuses on human ILC3, does also show the gain of cytotoxicity in human tonsillar $CD94^+$ ILC1 in response to Il12. We would like to point out, that this report shows that these cells partially express Eomes as well as RORγt, raising the possibility that the cultures contain NK- and ILC3-like cells. Indeed, the reported gain in cytotoxicity from these $CD94^+$ ILC1 was associated with Eomes, suggesting that these findings do not reflect the differentiation of ILC1s that we analyzed here. We are nevertheless intrigued by the finding that human ILCs have cytotoxic function, reinforcing the relevance of our findings. We have discussed the reviewer's point in the revised manuscript:

“Of note, the acquisition of cytotoxic function has been previously documented in in vitro cultures of human tonsillar CD94+ ILCs in response to Il12 (Krabbendam et al., 2020). Given that these cells do not express Hobit, and that this differentiation included the upregulation of Eomes, it is unclear whether it represents the human correlate of the differentiation of committed $Eomes^-$ ILC1 that we have investigated here. Hobit is, however, broadly expressed in human NK cells, intestinal intraepithelial ILC1s as well as subsets of activated effector T cells (Collins et al., 2019; Vieira Braga et al., 2015). Therefore, beyond its reported function in tissue-residency, Hobit may be a critical differentiation factor of these type-1 polarized lymphocytes, consistent with its role in ILC1s that we identified here. It will be

important to investigate the transcriptional programs regulating expansion potential versus effector differentiation in human Hobit+ versus Hobit- ILCs and NK cells."

Minor comments
- Why is the surface receptor CD127 stained intracellularly? Please clarify.

CD127 is expressed at low levels and can be further downregulated upon binding Il7, as also highlighted by the reviewer. To account for that, we have stained it intracellularly in our analyses (e.g. in the culture experiments), or we have amplified the signal through Biotin-Streptavidin for sorting. We have carefully validated these protocols. Consistent correlation of protein and mRNA expression is also confirmed by CITE-Seq in Figure 1c. We have provided a clarification of the rationale for intracellular CD127 staining in the materials section.

- The observed change in cellular state is not functionally tested. Do cytotoxic ILCs have the capacity to kill? This needs to be demonstrated, for example using K562 cells.

K562 are human target cells used for human NK cell assays. We have addressed the reviewer's request using an experimental setup adequate to the murine system. In line with the expression of granzymes, isolated $CD127^-$ ILC1 displayed a higher ability to kill YAC target cells than $CD127^+$ ILC1 (new Figure 1h). Therefore, the labeling of $CD127^-$ ILC1 as cytotoxic-like ILC1 appears justified.

- Extended data 1b (2nd and 3rd panel) axis incorrectly annotated.

We apologize for the mistake, which we have corrected (Extended Data Figure 1b).

- "….defining them as bona-fide ILC1…." Here the figure# should be added.

We have added the figure number to clarify.

- Why are MLN-derived ILC2 and ILC3 used as controls and not controls from the same tissue (extended figure 1d)?

Distinct from human livers, ILC2 and ILC3 represent very rare populations in healthy murine livers at steady state. We have performed parallel analyses of the mesenteric LNs, which contain all major ILC subsets, as positive controls and for validation of our stains. We have included the original liver data for these populations in the revised manuscript (new Extended Data 4d).

- The authors use OP9-DL1 cells for culturing ILC1s. Have the authors used OP9-Neo to control for biased expansion towards "cytotoxic" ILC1s as a consequence of constitute Notch signaling?

As mentioned above, we have included this control in the revised manuscript. It does not appear that Notch signaling impacts on ILC1 differentiation in our *in vitro* culture system (new Extended Data 3d).

Reviewer #2

In this article, the authors used various mouse models, single-cell analyses, in vitro culture and adoptive transfer experiments to show the existence of three stages of differentiation along the ILC1 developmental pathway. These three stages include cKit+CD127+Tcf7high, cKit-CD127+Tcf7int and cKit-CD127-Tcf7- cells, all stages expressing NK1.1 and therefore being already committed to the ILC1 lineage. Moreover, they show that the transcription factor Hobit is required for ILC1 differentiation to the TCF7int and TCF7low stages. Altogether, these data are novel and original and this paper is therefore a good candidate for publication in NI.

We thank the reviewer for the positive assessment of our work and for pointing out the novelty, relevance and potential impact of our study.

Main points

1) In their previous study (Zeis et al, Immunity 2020), the authors identified circulating ILCPs in the mouse lung. Their phenotype appears strikingly similar to that of early-stage ILC1s identified here, with the exception of NK1.1. Thus, it would be useful to determine how TCF7high ILC1s compare to ILCPs in terms of global gene expression patterns. Are there circulating ILCPs in the liver?

We are also intrigued by the "progenitor genes" that we identified in the Tcf1^{hi} "early" ILC1. Nevertheless, these cells can be clearly delineated and appear downstream of ILCPs based on expression of NK1.1, NKp46, Tbet, Hobit and the ability to produce IFNγ upon stimulation, as we already showed in the original manuscript.

We do agree that a comparative analysis of gene expression, as suggested by the reviewer, is insightful to substantiate the comparison between ILCPs and early, committed ILC1s. Therefore, we have integrated such a comparison as new Figure 6a-d and Extended Data 5d-g, in the respective section in the manuscript reads:

"The markedly increased expression of Tcf7, Rora, Il18r1 and Kit, urged us to address whether WT or Hobit-deficient Tcf1^{hi} early ILC1s resembled ILCPs. To this end, we selected a list of ILCP signature genes based on several recent studies(Harly et al., 2019; Walker et al., 2019; Xu et al., 2019; Zeis et al., 2020) and calculated expression scores in the ILC1 clusters identified in our single cell mRNA-Seq analysis. These comparisons yielded a low ILCP score for all hepatic ILC1 subsets but readily identified ILCPs in a dataset recently published by Harly et al(Harly et al., 2019) (Fig. 6a-d). The unbiased derivation of an ILCP core signature from this dataset yielded similar results (Extended Data Figure 5c-f). Consistently, while some "ILCP genes" were indeed enriched in Tcf1^{hi} early ILC1s, they largely lacked expression of other genes associated with ILCPs, such as Tox2, Zbtb16, Bcl11b, Pdcd1, Lef1, Myb and Rxrg. Instead, all Hobit-deficient and sufficient ILC1 subsets expressed ILC1 marker genes, that were not detected in ILCPs. As in WT mice, Hobit-deficient Tcf1^{hi} ILCs had a CD200r^{+} Tbet^{+} Rorγt^{-} Hobit^{Tom+} phenotype and expressed IFNγ upon stimulation (data not shown). These data demonstrate that Hobit does not contribute to the commitment of ILCP to the ILC1 lineage but specifically acts in lineage committed ILC1 to regulate their effector differentiation."

As requested by the reviewer, we have analyzed the presence of putative ILCPs in the liver. We have detected cells with a phenotype consistent of ILCPs in the liver (new Figure 4e, g). We have also detected liver-resident LSM cells that have been proposed to contribute to the local differentiation of ILC1 while our manuscript was under revision(Bai et al., 2021). Analysis of Hobit-reporter and lineage-tracer mice to delineate at which stages of ILC development Hobit expression is initiated shows that Hobit expression first appears in Tcf1^{hi} ILC1 and is absent from all putative upstream progenitors to ILC1s, and all other types of ILCs that we have analyzed (new Figure 4d, f-m):

"Interestingly we found that expression of Hobit was specifically acquired at the stage of Tcf1^{hi} ILC1s (Fig. 4c). Hobit expression was absent in ILCPs in the bone marrow and in Lin- CD127^{+} NK1.1^{-} liver cells, which contain hepatic ILC2 and ILC3 as well as cells with a cKit^{+} Il18r1^{+} Tcf1^{hi} phenotype characteristic of circulating and tissue-associated ILCPs(Ghaedi et al., 2020; Lim et al., 2017; Zeis et al., 2020). We also failed to detect Hobit reporter activity in recently discovered liver-resident lineage^{-}

Sca1^{+}Mac-1^{+} (LSM) cells that may contribute to the local differentiation of ILC1(Bai et al., 2021) (Fig. 4d-f). To test the developmental potential of Hobit-expressing cells, we took advantage of the Hobit-driven Cre recombinase of Hobit reporter mice by crossing these mice onto ROSA26-flox-stop-flox-eYFP (ROSA26-eYFP) mice. The Hobit-driven Cre recombinase will excise the floxed transcriptional stop sequence and enable fate mapping of Hobit expression through constitutive eYFP expression. Previous analysis has shown the efficacy and specificity of these Hobit lineage tracer mice in T cells(Behr et al., 2020). Both Hobit reporter activity and Hobit fate mapping were largely absent in ILC lineages other than ILC1 in the liver (Fig 4h-j). As ILC2 and ILC3 are relatively rare cells in the liver, we confirmed these findings in the mesenteric LN and small intestine, and for ILC2 in lung and ILC3 in the intestine (Fig. 4k-m and data not shown). These data demonstrate, that our analysis identified Tcf1^{hi} early yet lineage-committed differentiation stages of effector ILC1s."

2) One possibility could be that ILCPs turn on T-bet expression as soon as they enter the liver (due to local microenvironments) and therefore quickly engage into the ILC1 program. But these cells are arguably still at an early stage of differentiation. This raises the question of their pluripotency. Can they differentiate into other ILC lineage when placed in the appropriate environment?

We agree with the reviewer that it is important to determine when ILC1 establish lineage commitment. As detailed above under point 1 of this reviewer, we have performed several new analyses including Hobit reporter and fate mapper mice (new Figure 4). Our data indicate that Hobit^{+} ILC1 do not significantly contribute to NK cells and other ILC lineages during development and homeostasis, suggesting that Hobit expression marks cells that are committed to the ILC1 lineage.

3) CD127+ CD161+ ILC1s were originally described in the human gut. Have the authors looked at the phenotype of mouse gut ILC1s? Do they only find CD127+ ILC1s as well? Studying the phenotype of ILC1s in lymphoid organs (lymph nodes, spleen, bone marrow) would be also important.

As also mentioned in response to comments of reviewer 1, we have extended our analysis of the ILC1 differentiation pathway to other organs including gut and mesenteric lymph node, and we provide analysis of splenic ILC1 as R7. Our data supports the identification of the same maturation stages of ILC1 in these tissues, but with organ-specific composition of the identified ILC1 subsets, as reported in our initial submission for liver, salivary gland and kidney. Indeed, as anticipated by the reviewer, the mouse gut largely contains CD127^{+} ILC1s similar to the mesenteric lymph nodes and the kidneys (new Figure 7). We show that expression of Tcf1, Il18r and Gzms is regulated by Hobit in these cells (new Figure 7 and Extended Data 6j-q, 7e,f). Importantly, ILC1 in these tissues have the capacity to differentiate into CD127^{-} Gzma/b^{hi} ILCs in *in vitro* cultures (new Figure 8d-f) and in an *in vivo* inflammatory model (new Figure 8g and Extended Data 8f), as detailed below under point 5 of this reviewer.

4) Throughout the paper, the authors refer to CD127+ ILC1s as "helper" and CD127- ILC1s as cytotoxic. I grant that previous studies documented this point, but I think it would be important to confirm it in the present study ie perform cytotoxicity assays and measure the cytokine secretion potential of both ILC1 stages.

We have included experiments demonstrating the cytotoxic potential of isolated populations of CD127^{+} and CD127^{-} ILC1. We have also included additional data assessing the capacity to produce IFNγ, TNF-α and GM-CSF (new Figure 1g).

As detailed in the response to reviewer 1, we would like to emphasize that we consider cytokine production a central effector function of all ILC1s that is maintained during the gradual differentiation towards cytotoxic subsets. To make this clearer, we have included new data showing IFNγ, TNFα and GM-CSF production by both CD127^{+} and CD127^{-} ILC1 (new Figure 1g). Consistent with the gradual changes observed in gene expression (Figure 2a and 3c), these data suggest that CD127^{+} ILC1 differentiate towards CD127^{-} ILC1 by gradually acquiring additional effector functions while forfeiting their expansion potential, reminiscent of the effector differentiation of cytotoxic T cells.

5) The present paper is limited to the description of ILC1 differentiation at steady state. I think it would be important to provide at least some data on how this differentiation is impacted in pathological situations (ie inflammation, cancer or infections).

As highlighted in the response to reviewer 1, we have included new analyses to address this point, the respective new section reads:

"These data suggested that these ILC1s can undergo further effector differentiation but may be "halted" in their differentiation due to the lack of activating signals or the active suppression in their respective tissue-niches. To test whether ILC1s can undergo further effector differentiation in these organs in vivo we performed depletion of regulatory T (Treg) cells, which creates an inflammatory setting to which ILC1 respond locally, as tissue-resident cells(Gasteiger et al., 2015) Intriguingly, we found that Treg cell depletion promoted the effector differentiation of ILC1s in all analyzed tissues, as evidenced by the downregulation of Tcf1 and the concomitant increase in Gzma and Gzmb expression (Fig. 8g and Extended Data Fig. 8h)."

These findings confirm the *in vivo* differentiation potential suggested by our previous analyses and are consistent with the idea that ILC1s are actively retained in their helper-like state in some tissues during homeostasis, but can gain additional effector functions in the absence of Treg control, or as a consequence of tissue inflammation. We believe that these new analyses provide a very exciting *in vivo* validation of the concepts emerging from our study.

Reviewer #3

This study identifies two subsets of hepatic ILC1, which vary with respect to their gene expression. CD127+ ILC1 are less mature and can differentiate and become Gzm+ ILC1. The authors examine mice deficient for the Hobit, a known transcription factor essential for the development of hepatic ILC1. Hobit KO mice fail to generate Gzm+ ILC1 whereas CD127+ ILC1 still develop. Finally, the salivary gland and kidney contain mainly CD127+ immature ILC1, which can be further differentiated in vitro. The study provides a detailed description of hepatic ILC1 development and the experiments are carried out to a high standard.

We thank the reviewer for the positive comments on our study.

However, the following points have to be addressed before the manuscript can be considered for publication.

Figure 1:
Did the authors exclude the possibility that CD127+ ILC1 are derived from ILC2 or ILC3?

We have explored whether CD127$^+$ ILC1 develop downstream of ILC2 and ILC3 with the use of Il5 fate mappers (to detect ILC2 offspring(Nussbaum et al., 2013) and RORγt fate mappers (to detect ILC3 offspring(Vonarbourg et al., 2010)).

Our data suggest that there is no significant contribution towards ILC1 from the ILC2 lineage at steady state (new Figure R8 and 9).

Both, CD127$^+$ and CD127$^-$ ILC1 subsets, but not NK cells, exhibited a significant degree of fate-mapping for Rorc expression, which can identify ILC1s that have differentiated from ILC3s(Bernink et al., 2013; Vonarbourg et al., 2010). Importantly, we and others have observed stochastic expression of Rorc in BM ILCPs(Walker et al., 2019; Zeis et al., 2020), which we also observed in the ILCP dataset of Harly *et al*(Harly et al., 2019). Therefore, ILC1 may also acquire RORc-fate-mapping during development. Consistent with this idea, the frequency of Rorc-FM was highly similar in ILC1s across different organs, and in the "immature" ILC1s found in the livers of Hobit KO mice (Figures R9 and new Extended Data Figure 2d, 4d). Importantly, both FM$^+$ as well as FM$^-$ ILC1 showed a similar pattern of CD127, Il18r1 and granzyme expression (Extended Data Figure 2e). We feel that these observations suggest that ILC1s acquire Rorc-FM during development. Independently thereof, our data suggest that both RORc-FM$^+$ and RORc-FM$^-$ ILC1s undergo a Hobit-dependent differentiation into CD127$^+$ Gzm^{lo} and CD127$^-$ Gzm^{hi} effector subsets.

As we feel that these are significant findings, we suggest to include these new data into the manuscript:

"Both, CD127$^+$ and CD127$^-$ ILC1 subsets, but not cNK cells, exhibited a high degree of fate-mapping for Rorc expression, which can identify ILC1s that have differentiated from ILC3s(Bernink et al., 2013; Vonarbourg et al., 2010), or from subsets of ILCPs marked by stochastic expression of Rorc(Walker et al., 2019; Zeis et al., 2020). This fatemapping analysis suggests that CD127$^+$ and CD127$^-$ ILC1 subsets have a shared developmental origin, while being separate from cNK cells. Conversely, both FM$^+$ as well as FM$^-$ cells showed a similar pattern of CD127, Il18r1 and granzyme expression (Extended Data Fig. 2d, e)."

and:

"Importantly, the few ILC1 detectable in Hobit KO mice were not "ex-ILC3", as they showed the same level of Rorc-fate-mapping as ILC1s in wt livers (Extended Data Fig. 4d)."

Is there a function for Gzm in ILC1?
Are Gzm+ ILC1 more cytotoxic towards target cells?
Can the authors demonstrate a non-redundant function for Gzm+ ILC1?

This point overlaps with point 4 of reviewer 2. As mentioned above, we have supported our definitions of helper and cytotoxic ILC1 with functional *in vitro* experiments on the isolated ILC1 subsets.

We have included new experiments to assess the cytotoxicity of CD127^{+} Gzm^{lo} versus CD127^{-} Gzm^{hi} ILC1 against YAC1 target cells, as suggested by the reviewer (new Figure 1h). These analyses support the elevated cytotoxic potential of CD127^{-} Gzm^{hi} compared to CD127^{+} Gzm^{lo} ILC1.

We think that addressing the functional relevance of granzymes in ILC1 is beyond the scope of our current manuscript, which focuses on the differentiation pathway of ILC1. Our mRNA and protein expression data show that ILC1s express a range of granzymes (A, B, C) that may have both unique and redundant functions. Moreover, genetic systems to specifically address these granzyme-driven cytotoxic functions of ILC1 are, to our knowledge, currently not available.

As highlighted by Reviewer 1, Gzmc+ ILC1 have been implicated in the control of tumors by Dadi *et al.*(Dadi et al., 2016); we had already included this reference in the introduction, and had highlighted presence of Gzmc mRNA. We now have added protein expression data for Gzmc in the revised Figure 1f).

Figure 2:
This could be a Suppl. Figure since these experiments are not central to the main conclusions. Stats are missing.

We have included additional datasets in the revised manuscript at the expense of additional space. As suggested by the reviewer, we have repositioned this figure in the supplementary figures (now Extended Data Figure 2). As requested, statistical analysis has been added to all figure panels.

Figure 3/4: How do the authors exclude that the two subsets of hepatic ILC1 identified have different functional specializations rather than being precursor and effector cells?

We do not think that the lineage relationships (“stem-like” versus “terminal” differentiated effector cells) and the functional specializations (helper versus cytotoxic) are mutually exclusive in the identified ILC1 subsets. As we have discussed in the manuscript, our findings are reminiscent of memory T cells, where Tcf1^{hi} progenitor-like or CM-like cells with high proliferative potential generate a progeny of effector cells with increased cytotoxic potential, yet still express a range of effector molecules themselves.

We had already shown in the manuscript that Tcf1^{hi} ILC1 are upstream of Tcf1^{lo} Gzm^{+} ILC1 and that both (in fact: all three identified) subsets produce IFNγ whereas CD127^{-} ILC1 express increased levels of granzymes and additional effector molecules, and are more cytotoxic (new Figure 1h and 4). These findings clearly suggest that all of the identified ILC1 subsets have functional roles. We have further substantiated these findings using analysis of proliferative potential (Extended Data 3f), cytokine production (new Figure 1g and R6) and cytotoxicity (new Figure 1h) of the ILC1 subsets. We have added novel comparisons of Tcf1^{hi} ILC1 to ILCPs, as well as Hobit fate-mapping data that support the view that these cells represent lineage-committed effector cells (new Figures 4h-m, 6 and Extended Data Figure 5d-g).

Is there a role for IL-2 in promoting Gzm+ ILC1 in vivo?

This is an interesting suggestion of the reviewer. Indeed, our *in vitro* cultures suggest that Il2 drives substantial expansion and expression of Gzms in ILC1 (Figure 2c). The finding that depletion of Foxp3^{+} Treg cells, which can suppress effector cells by regulating availability of Il2, are also consistent with a role of Il2 in promoting Gzm^{+} ILC1. To directly address the question of the reviewer, we have generated NKp46^{Cre} x CD25$^{fl/fl}$ mice. The setup of these mice allowed us to address the role of Il2 in the regulation of granzyme expression in ILC1. We found that only few ILC1 expressed CD25 at steady state. Conversely, we found no difference in the differentiation state and expression level of granzymes (Figure R5).

Our *in vitro* stimulation, however, suggests that inflammatory cytokines Il12/18 induce the upregulation of CD25, and therefore raises the possibility that Il2 may play a role in activating e.g. $CD127^{+}$ $Il18r1^{+}$ "helper-like" ILC1 and driving their expansion and differentiation towards Gzm^{hi} effector cells. We have generated preliminary data with the injection of CD122-targeting Il2-complexes which triggered high Gzm expression in NK cells, but did not dramatically boost granzymes in ILC1. Therefore, Il2 may be one of an array of signals coregulating Gzm expression in ILC1s, and understanding these signals *in vivo* will require novel genetic tools and significantly more work. We feel that these represents important future research questions.

We have highlighted our findings from the *in vitro* cultures to suggest these possibilities to the community:

"Our in vitro cultures suggest that Il2 and Il15 can contribute to the transition of helper-like ILC1 into cytotoxic-like ILC1. Il2 may be more relevant for ILC1 differentiation under inflammatory conditions as occurs upon depletion of regulatory T cells, while Il15 may dominate under homeostatic conditions, as supported by its essential contribution to ILC1 development(Daussy et al., 2014; Klose et al., 2014)."

Figure 5:
Which molecules regulate Hobit expression in ILC1?

As also detailed in our response to reviewer 1, our work about the function of Hobit suggests that unraveling the instructive cues of Hobit regulation will further improve our understanding of the final differentiation steps of ILC1. Therefore, we have tested the role of known regulators of Hobit expression that were previously implied to regulate Hobit in tissue-resident $CD8^{+}$ T cells.

To this end, we performed *in vivo* neutralization experiments of Il15 to analyze its influence on ILC1 effector diversification, as suggested by the reviewer (Figure R4). Furthermore, we have analyzed mice deficient in STAT4 (an essential signaling molecule for Il12), mice deficient in IL18r1 and mice deficient in Il2 ($NKp46^{Cre}$ x $CD25^{fl/fl}$ mice) for effector differentiation of ILC1 (Figures R2, R3, and R5). Our analyses therefore indicate that, under steady state conditions, Il12, Il18, Il2 and Il15 (beyond it's essential function during ILC1 development (Daussy et al., 2014; Klose et al., 2014) are not required to regulate the differentiation of ILC1 into the effector subtypes that we have identified in this study. As we show that this differentiation requires Hobit (Figure 5), we conclude that these cytokines are not required to induce Hobit expression.

Conversely, Cortez *et al* have previously shown that ILC1s in the liver develop independently of TGFβ to normal numbers and have unaltered expression of CD49a, CD69 and CD73 in Ncr1-Cre x Tgfβr2-floxed mice(Cortez et al., 2016) (Cortez et al., Immunity 2016, Fig. S1). As we demonstrate that numbers of ILC1s in the liver as well as expression of these genes are significantly reduced in Hobit KO mice, we conclude that TGFβ is not required to drive Hobit expression.

In an additional attempt to address the reviewers question, we have sorted BM ILCPs and performed established *in vitro* differentiation assays that we have previously established in the lab(Zeis et al., 2020) to determine whether cytokines Il7, Il2, Il15, Il12, Il18 and TGFβ, or combinations thereof, are able to induce Hobit expression. We have succeeded in differentiating $CD49a^{+}$ $Tbet^{+}$ $Eomes^{-}$ ILC1-like cells, however, these cells did not upregulate Hobit as assessed with the Hobit-reporter and fate-mapper allele (data not shown).

Our data therefore indicate that additional factors, likely from within the respective tissue niches, are required to instruct Hobit expression. We feel that the main focus of our manuscript is the function of Hobit in driving ILC1 effector differentiation, and that unraveling the instructive cues of Hobit will require future work that goes beyond the current focus of the manuscript. As we are already reaching the limit of the Extended Figure format, we propose to not include these analyses. Nevertheless, we have included a section in the discussion to account for the reviewer's suggestion in the manuscript:

"The instructive signals driving the differentiation of committed ILC1 in vivo remain unclear. Our in vitro cultures suggest that Il2 and Il15 can contribute to the transition of helper-like ILC1 into cytotoxic-like ILC1. Il2 may be more relevant for ILC1 differentiation under inflammatory conditions as occurs upon

depletion of regulatory T cells, while Il15 may dominate under homeostatic conditions, as supported by its essential contribution to ILC1 development(Daussy et al., 2014; Klose et al., 2014). In preliminary analyses, the phenotype of liver ILC1s was not altered in STAT4-KO and Il18R1-KO mice, or when we neutralized Il15 (data not shown). Similarly, hepatic ILC1s had no detectable change in phenotype when lacking Tgfbr2(Cortez et al., 2016). These observations suggest that these cytokines do not directly maintain or induce Hobit and highlight that additional work will be required to address the instructive signals of Hobit expression and the differentiation of lineage-committed ILC1."

We hope the reviewer agrees that the main focus of our manuscript is the function of Hobit in driving ILC1 effector differentiation, and that unraveling the instructive cues of Hobit will require substantial future studies that go beyond the focus of the current manuscript.

Are Il18r1, c-kit, Tcf7, and GzmA/B direct target genes of Hobit?

We have previously addressed direct target genes of Hobit in CD8 T cells. We could show that *Tcf7* is a direct target gene of Hobit, but in these experiments we have found no evidence for a direct role of Hobit in the regulation of the other genes mentioned by the reviewer(Mackay et al., 2016). With the currently available experimental setup (overexpression of tagged Hobit in cell cultures, given that no suitable murine Hobit antibody is available for ChIPSeq), it will not be possible to obtain sufficient quantities of ILC1 to directly perform ChIPSeq analysis in this subset.

To address the reviewers request, we have included new analyses of these available ChIP Seq data, the respective section in the manuscript reads as follows:

"To identify putative targets of Hobit in ILCs we matched our gene expression data with Hobit chromatin binding sites established through Hobit ChIP-Seq in T cells (MacKay et al). Hobit has been suggested to directly regulate the expression of genes associated with T cell stemness (i.e. the capacity to expand and generate downstream effector cells), including Tcf7, Bach2 and Batf3(Ataide et al., 2020; Mackay et al., 2016; Yao et al., 2021). Interestingly, we found that expression of these genes was increased in Hobit-deficient ILC1 and was downregulated along the transition of ckit^{+} Tcf1^{hi} CD127^{hi} to cKit$^{\lambda}$ Tcf1^{int} CD127^{int} ILC1, consistent with the idea that Hobit regulates ILC1 "stemness" versus effector differentiation by modulating these genes (Fig. 6e, f)."

Figure 6: No statistics throughout except the final panels.

We have added statistics throughout the figure, now Figure 7

Figure 7:
What are the cues regulating Hobit expression in different tissues?

The instructive cues of Hobit expression are currently incompletely defined, see above. It is also not known whether these cues are identical throughout tissues or whether they act in a tissue-specific manner.

We would like to point out that essential *in vivo* cues for the regulation of Hobit expression have neither been identified for tissue-resident CD8 T cells or NKT cells. We feel that the focus of our work is the function of Hobit in driving ILC1 effector differentiation, and that unraveling the instructive cues of Hobit in a tissue-specific manner will require *in situ* transcriptomics of ILC tissue niches and novel genetic models, and therefore requires substantial future work that goes beyond the current focus of the manuscript.

Figure 8:
In which developmental stage is Hobit induced during ILC1 development? Is Hobit expressed in ILC1 or precursors before populating the liver?

As also detailed in our response to reviewers 1 and 2, we have dedicated two new Figures to address this important questions. The respective sections read:

"Tcf1^{hi} early ILC1s are Hobit-expressing lineage-committed effector cells

The Tcf1^{hi} early-stage ILC1s identified here expressed several genes that are also expressed in ILCPs and exhibited a marked expansion potential, raising the question whether these cells represent progenitor-like or lineage-committed cells. In contrast to ILCPs, cKit^{+} Tcf1^{hi} ILC1 expressed NK1.1, NKp46 and Tbet and produced IFNγ upon stimulation (Fig. 4a, b). Interestingly we found that expression of Hobit was specifically acquired at the stage of Tcf1^{hi} ILC1s (Fig. 4c). Hobit expression was absent in ILCPs in the bone marrow and in Lin- CD127^{+} NK1.1^{-} liver cells, which contain hepatic ILC2 and ILC3 as well as cells with a cKit^{+} Il18r1^{+} Tcf1^{hi} phenotype characteristic of circulating and tissue-associated ILCPs(Ghaedi et al., 2020; Lim et al., 2017; Zeis et al., 2020). We also failed to detect Hobit reporter activity in recently discovered liver-resident Lin^{-}Sca1^{+}Mac-1^{+} (LSM) cells that may contribute to the local differentiation of ILC1(Bai et al., 2021) (Fig. 4d-f). To test the developmental potential of Hobit-expressing cells, we took advantage of the Hobit-driven Cre recombinase of Hobit reporter mice by crossing these mice onto ROSA26-flox-stop-flox-eYFP (ROSA26-eYFP) mice. The Hobit-driven Cre recombinase will excise the floxed transcriptional stop sequence and enable fate mapping of Hobit expression through constitutive eYFP expression. Previous analysis has shown the efficacy and specificity of these Hobit lineage tracer mice in T cells(Behr et al., 2020). Both Hobit reporter activity and Hobit fate mapping were largely absent in ILC lineages other than ILC1 in the liver (Fig 4h-j). As ILC2 and ILC3 are relatively rare cells in the liver, we confirmed these findings in the mesenteric LN and small intestine, and for ILC2 in lung and ILC3 in the intestine (Fig. 4k-m and data not shown). These data demonstrate, that our analysis identified Tcf1^{hi} early yet lineage-committed differentiation stages of effector ILC1s.

And:

"The markedly increased expression of Tcf7, Rora, Il18r1 and Kit, urged us to address whether WT or Hobit-deficient Tcf1^{hi} early ILC1s resembled ILCPs. To this end, we selected a list of ILCP signature genes based on several recent studies(Harly et al., 2019; Walker et al., 2019; Xu et al., 2019; Zeis et al., 2020) and calculated expression scores in the ILC1 clusters identified in our single cell mRNA-Seq analysis. These comparisons yielded a low ILCP score for all hepatic ILC1 subsets but readily identified ILCPs in a dataset recently published by Harly et al(Harly et al., 2019) (Fig. 6a-d). The unbiased derivation of an ILCP core signature from this dataset yielded similar results (Extended Data Figure 5c-f). Consistently, while some "ILCP genes" were indeed enriched in Tcf1^{hi} early ILC1s, they largely lacked expression of other genes associated with ILCPs, such as Tox2, Zbtb16, Bcl11b, Pdcd1, Lef1, Myb and Rxrg. Instead, all Hobit-deficient and sufficient ILC1 subsets expressed ILC1 marker genes, that were not detected in ILCPs. As in WT mice, Hobit-deficient Tcf1^{hi} ILCs had a CD200r^{+} Tbet^{+} Rorγt^{-} Hobit^{Tom+} phenotype and expressed IFNγ upon stimulation (data not shown). These data demonstrate that Hobit does not contribute to the commitment of ILCP to the ILC1 lineage but specifically acts in lineage committed ILC1 to regulate their effector differentiation."

Together, these experiments delineate the developmental stage at which Hobit expression is initiated and confirm that this transcription factor marks lineage commitment of ILC1 across tissues. Our new analyses provide additional evidence for a conserved developmental trajectory of ILC1s across tissues and highlight that this differentiation potential is actively regulated *in vivo* in the respective tissue-niches and by inflammatory stimulation. Together, our findings establish a conceptual framework that connects tissue-specific phenotypes of ILC1 along a uniform differentiation pathway driven by Hobit.

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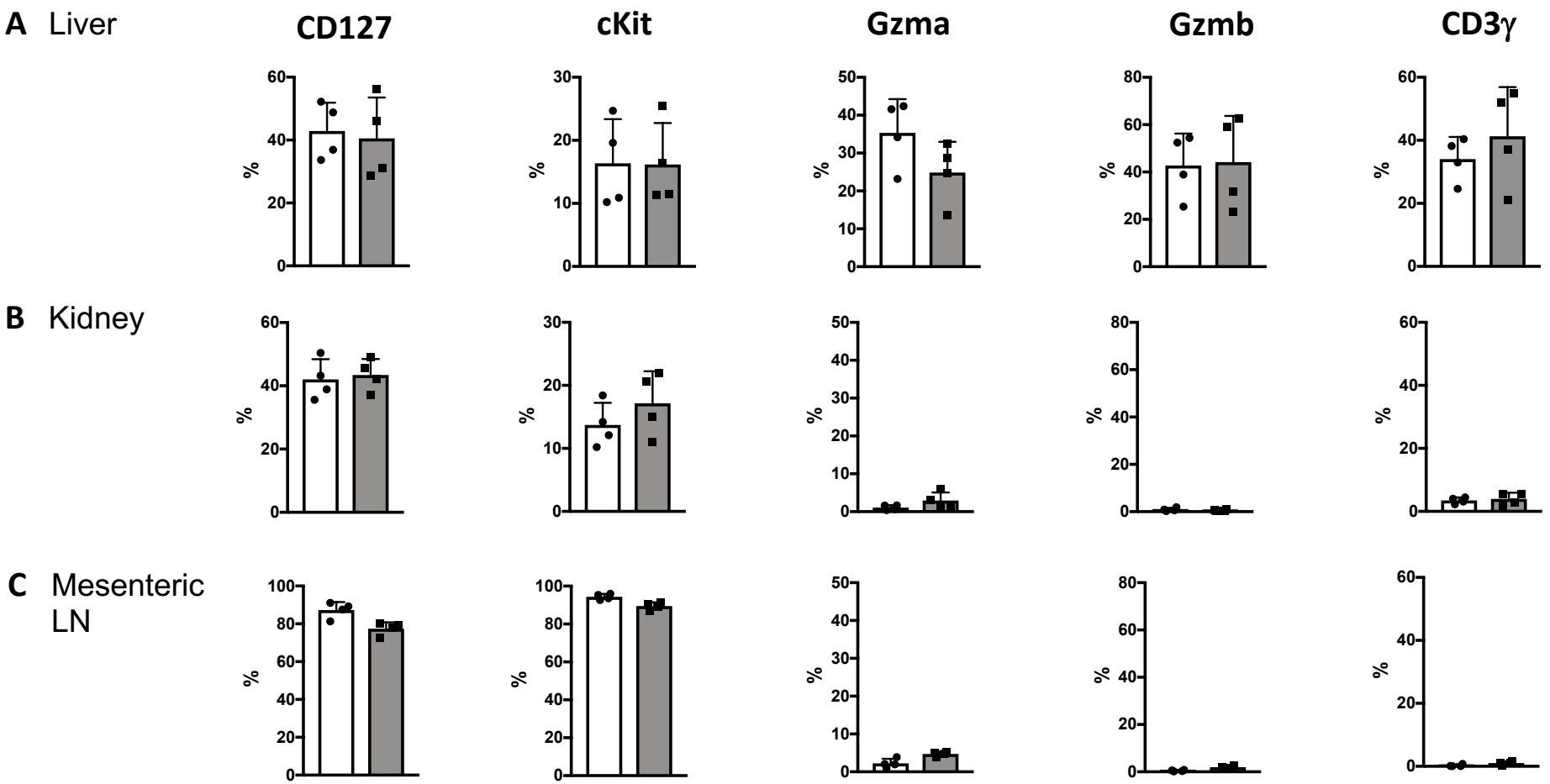
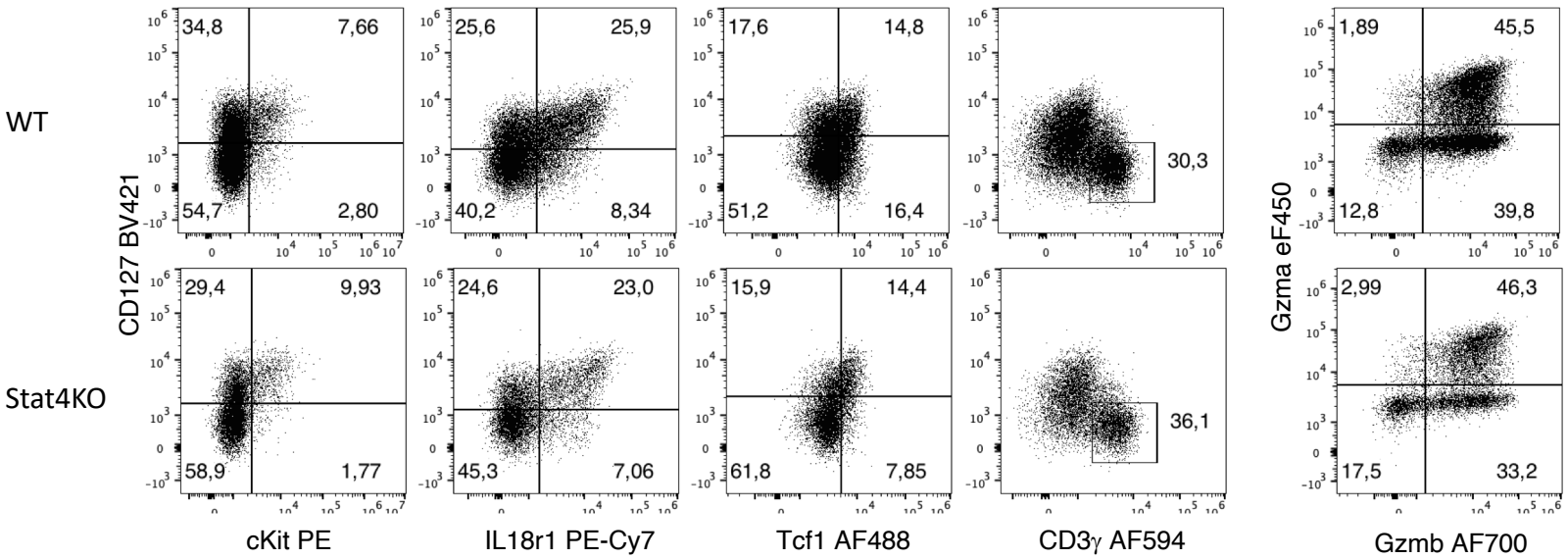
R1: Eomes$^{fl/fl}$ NKp46^{cre} mice



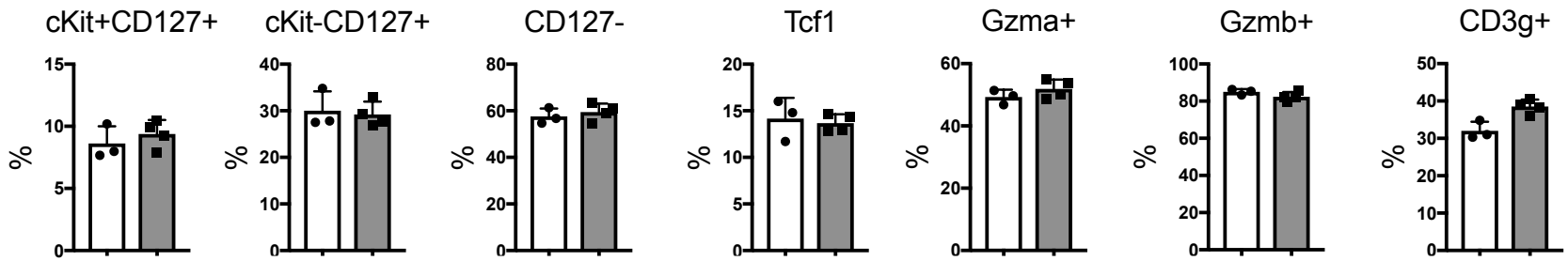
R1:
Frequency of ILC1s expressing indicated proteins in NKp46^{Cre} versus NKp46^{Cre} Eomes$^{fl/fl}$ mice in liver (A), kidney (B), and mesenteric lymph nodes (C).

R2: STA4-KO mice

A



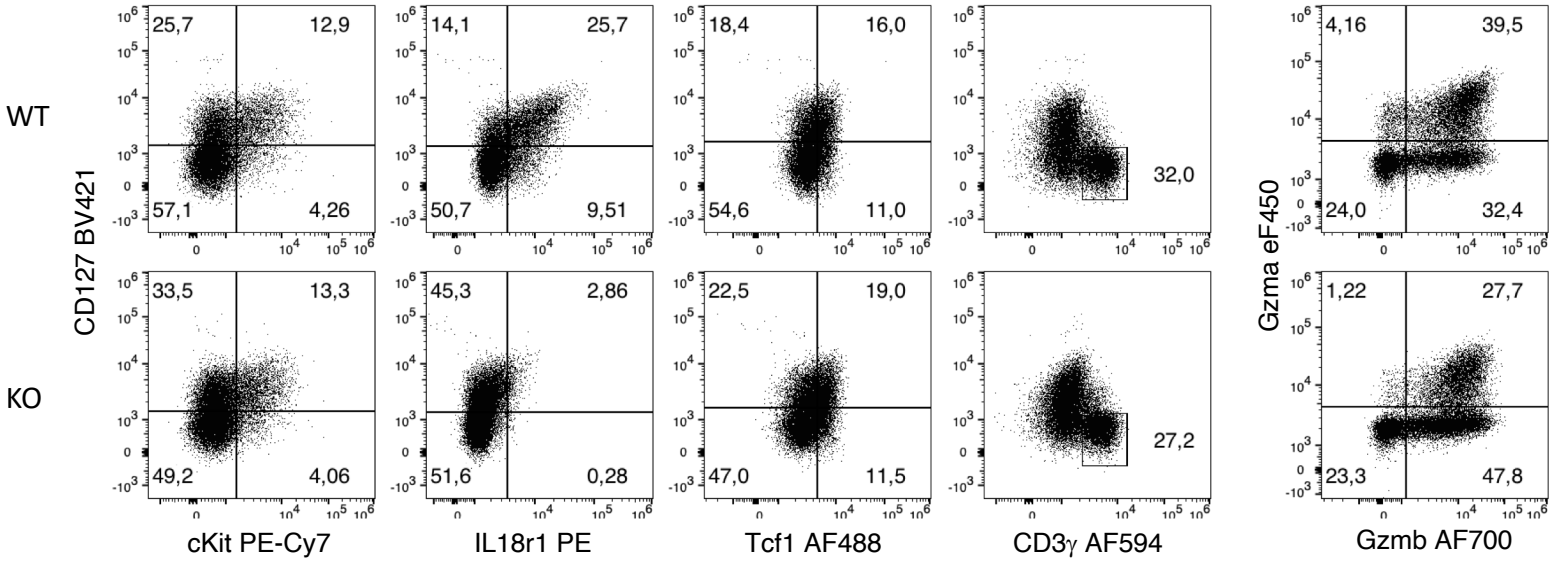
B



R2: Liver ILC1 effector differentiation is independent of Stat4 signaling.
Representative FACS analysis of indicated marker expression of ILC1 from livers of WT and Stat4-KO mice. Bar graphs represent frequency of ILC1s expressing indicated marker proteins of WT (white) and Stat4-KO (gray) mice.

R3: Il18r1-KO mice

A



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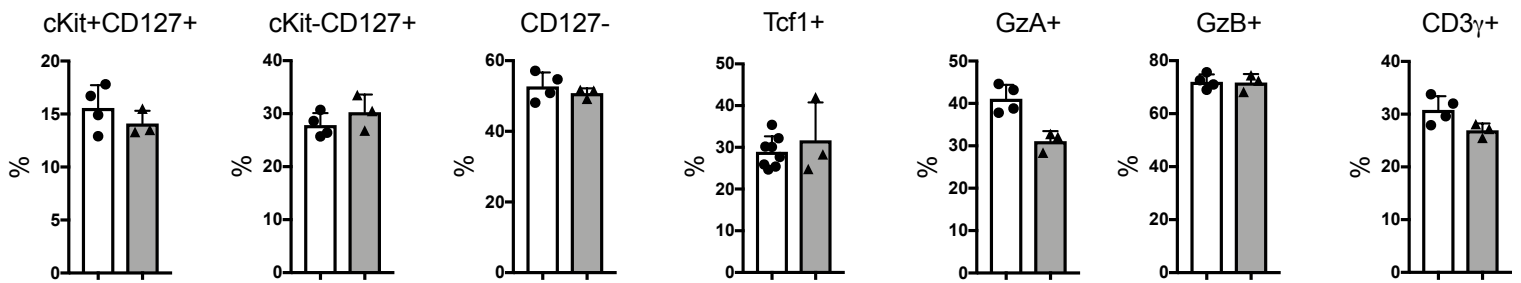
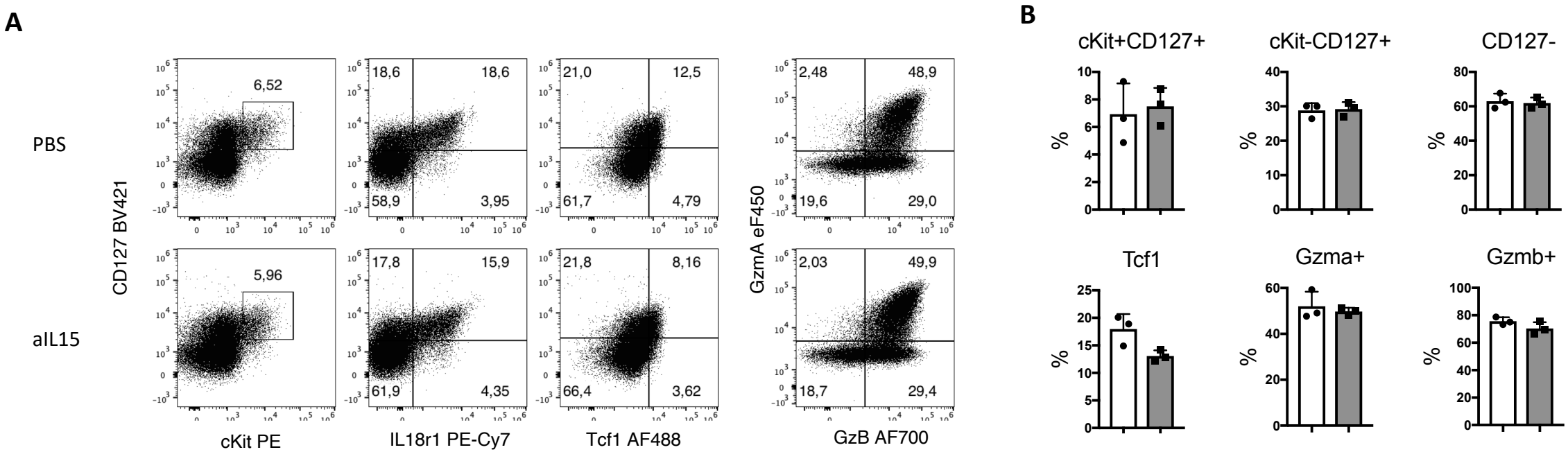


R3: Liver ILC1 effector differentiation is independent of Il18r1 signaling.
Representative FACS analysis of indicated marker expression of ILC1 from livers of WT and Il18r1-KO mice. Bar graphs represent frequency of ILC1s expressing indicated marker proteins of WT (white) and Il18r1-KO (gray) mice.

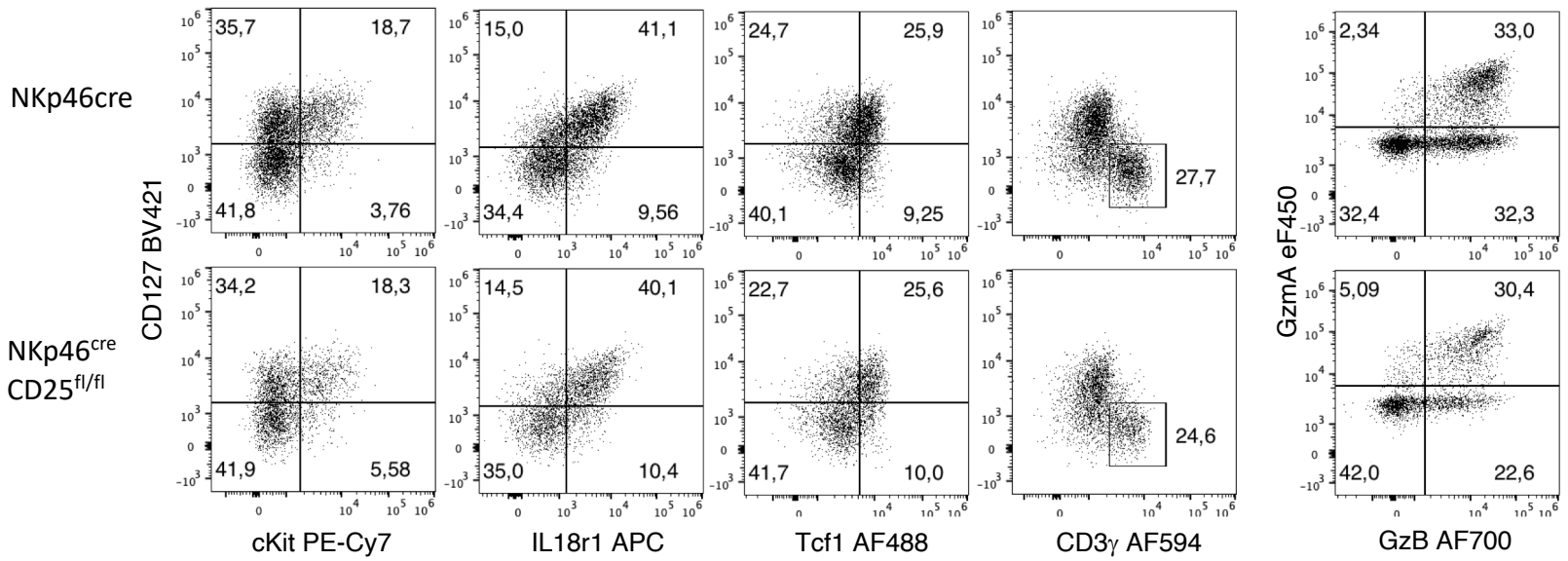
R4: IL15 neutralization



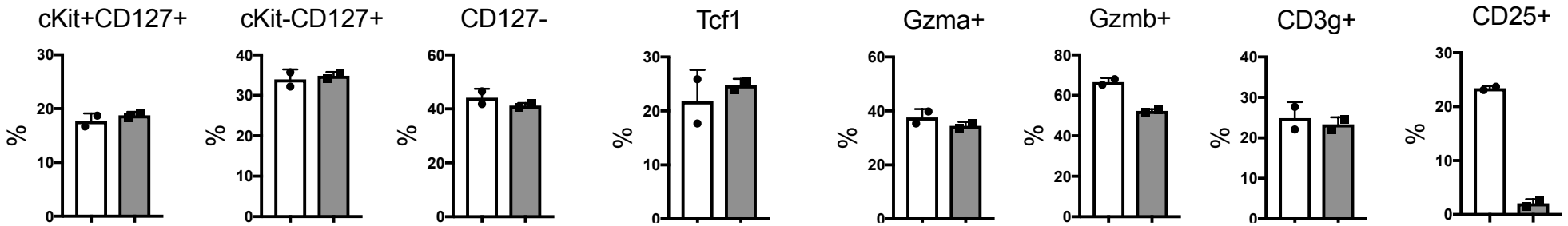
R4: Liver ILC1 effector differentiation is independent of Il15.
Representative FACS analysis of indicated marker expression of ILC1 from livers of WT mice treated daily intraperitoneally with 100 μg neutralizing anti-mouse Il15 antibody (clone AIO.3, BioXCell) in 200 ul PBS (aIL15) or PBS only (PBS). Mice were analyzed after 13 days of daily treatment. Bar graphs represent frequency of ILC1s expressing indicated marker proteins of PBS-treated (white) and aIL15-treated (gray) mice (B).

R5: NKp46^{cre} x CD25$^{fl/fl}$

A

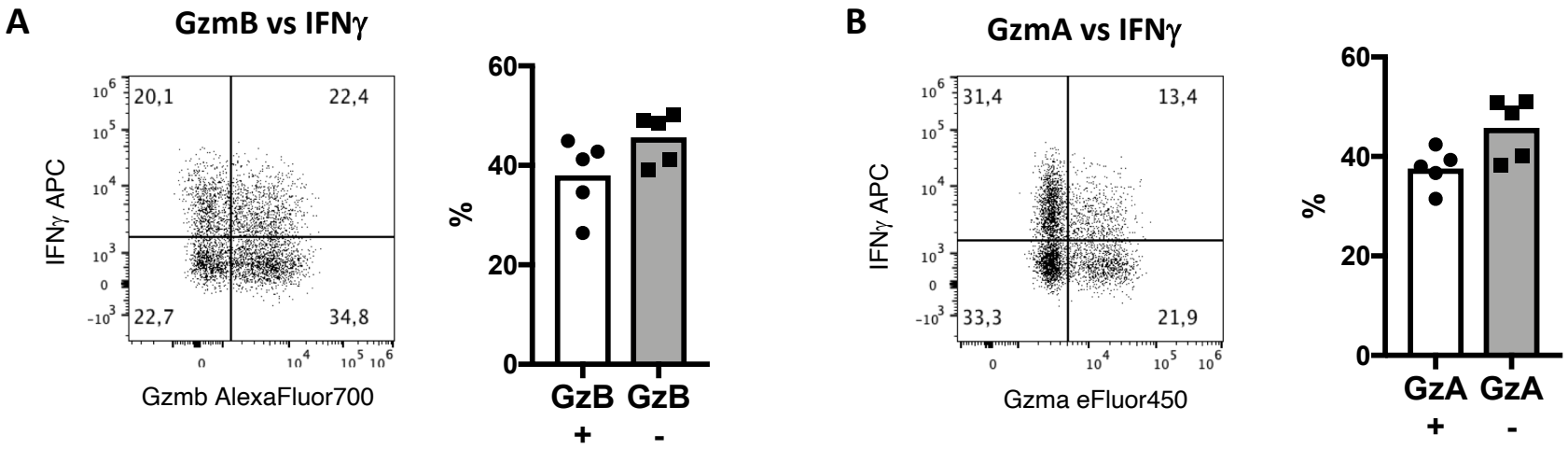


B



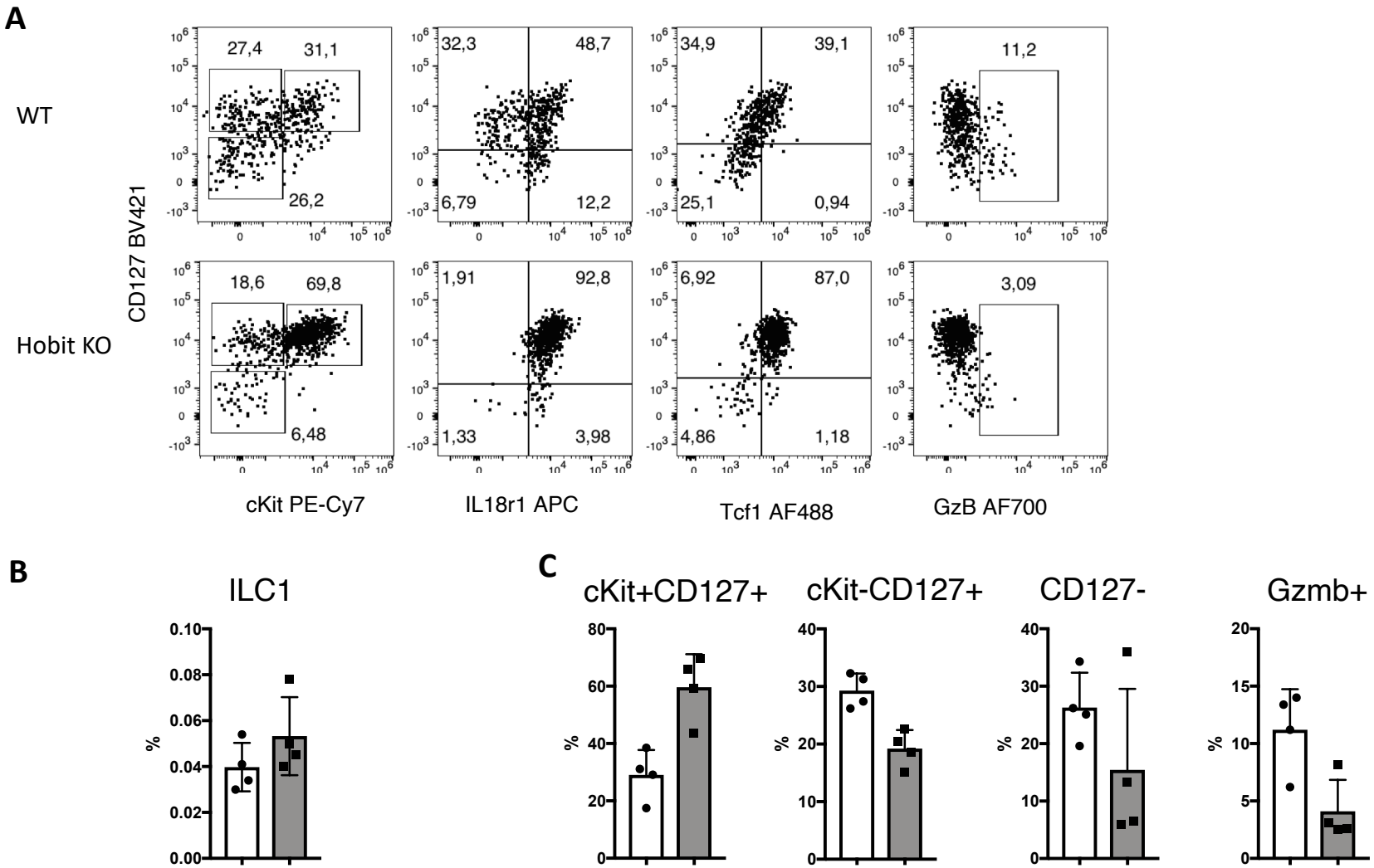
R5: Liver ILC1 effector differentiation is independent of Il2 signaling.
Representative FACS analysis of indicated marker expression of ILC1 from livers of WT (NKp46^{cre}) and NKp46^{cre} CD25$^{fl/fl}$ mice. Bar graphs represent frequency of ILC1s expressing indicated marker proteins of WT (white) and NKp46cre CD25$^{fl/fl}$ (gray) mice (B). CD25 detection after 4h *in vitro* stimulation with 10 ng/ml Il12 and 10 ng/ml Il18.

R6: IFNg vs Gzm



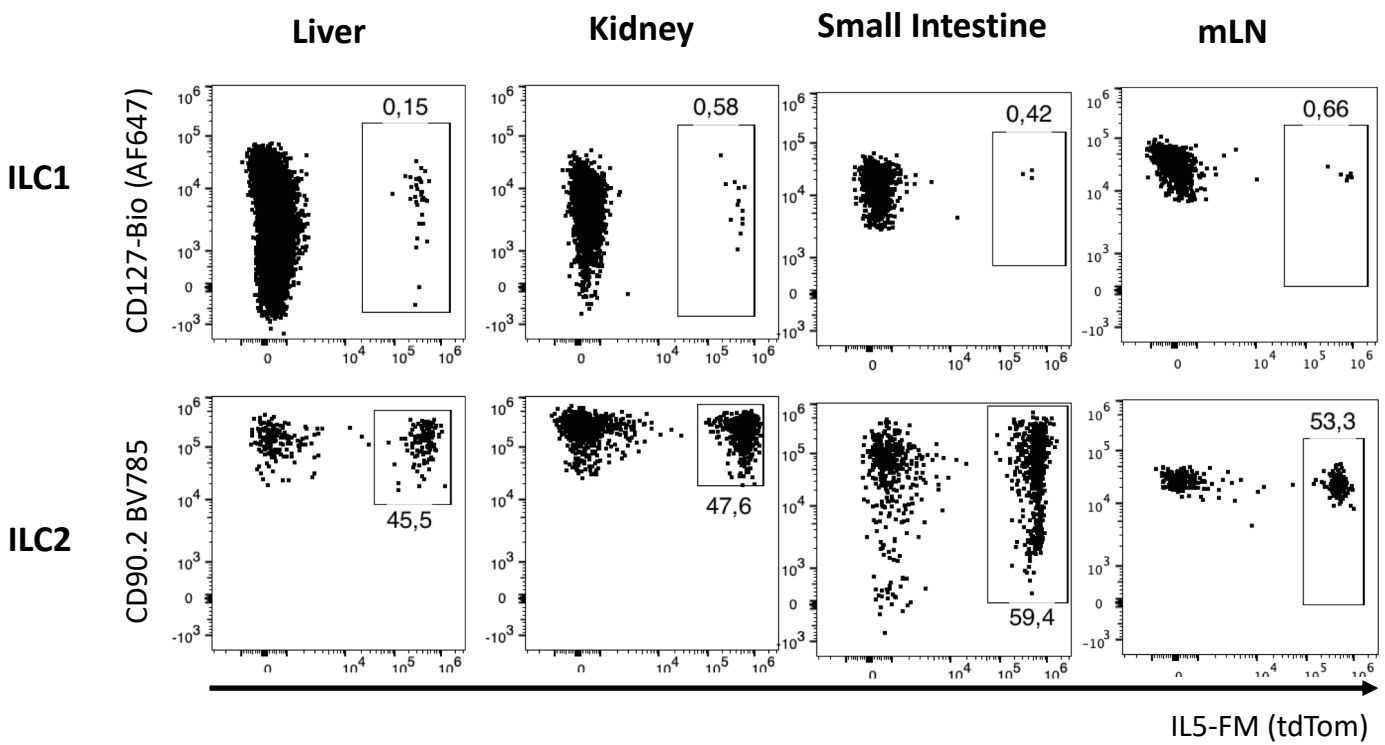
R6: IFNγ production by liver ILC1 is independent of Gzm expression.
Representative FACS analysis of indicated marker expression of ILC1 from livers of WT mice. Bar graphs represent frequency of IFNγ expressing cells within indicated Gzm-positive (white) or Gzm-negative (gray) cells. Data are representative of 2 independent experiments with n=5 mice per group.

R7: ILC1 phenotype in spleen



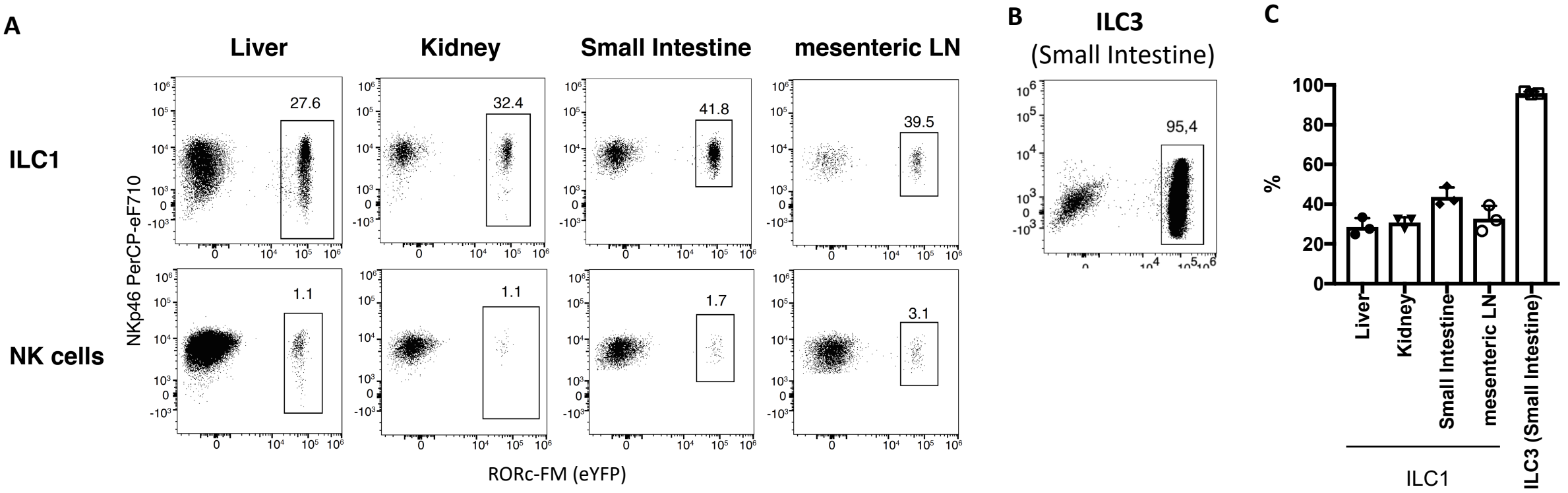
R7: Hobit drives the effector maturation of ILC1 in the spleen.
Representative FACS analysis of indicated marker expression of ILC1 from livers of WT and Hobit^{KO} mice. Bar graphs represent frequency of ILC1s within live CD45^{+} cells (B) and ILC1s expressing indicated marker proteins (C) of WT (white) and Hobit^{KO} (gray) mice.

R8: IL5^{Cre} Rosa^{Tom} mice



R8: ILC2 do not substantially contribute to ILC1 lineage across tissues.
Representative FACS analysis of IL5 fate map reporter signal (Tom) in ILC1 (top) and ILC2 (bottom) from indicated organs of IL5^{Cre} Rosa^{Tom} mice. ILC2 were gated as Lin^{-}CD127^{+}ST2^{+} cells (liver, kidney, mesenteric LN) or Lin^{-}CD127^{+}KLRG1^{+}CD25^{+} cells (small intestine) Data are representative of 2 independent experiments with n=3 individual mice.

R9: RORc^{Cre} Rosa^{eYFP} mice



R9: RORc-fate map labeling in ILC1 across tissues.
Representative FACS analysis of RORc fate map reporter signal (eYFP) in ILC1 (top) and NK cells (bottom) from indicated organs of RORc^{Cre} Rosa^{eYFP} mice. Frequency of fate map positive small intestinal ILC3 shown as control (B). Bar graphs represent frequency of RORc fate map positive cells within ILC1 of indicated organs. Data are representative of 2 independent experiments with n=3 individual mice.

Decision Letter, first revision:

Subject: Your manuscript, NI-A31400A

Message: Our ref: NI-A31400A

14th Jul 2021

Dear Dr. Gasteiger,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "Effector differentiation downstream of lineage commitment in ILC1 is driven by Hobit across tissues" (NI-A31400A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

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

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

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

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Effector differentiation downstream of lineage commitment in ILC1 is driven by Hobit across tissues". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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

Best regards,

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

On behalf of

Laurie A. Dempsey, Ph.D.
Senior Editor
Nature Immunology
l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X

Reviewer #1:
Remarks to the Author:
The authors adequately addressed the raised concerns and performed a substantial amount of additional experiments, further demonstrating their key finding that Hobit is essential for acquiring cytotoxic effector functions downstream lineage commitment in ILC1.

Reviewer #2:
Remarks to the Author:
The authors addressed all my previous comments, and the paper is now suitable for publication.

Reviewer #3:
Remarks to the Author:
I appreciate the quality of the data, and I do understand that this study is focused on ILC1 differentiation in tissue. However, given that Hobit was previously identified as a critical transcription factor for ILC1 development, I do not understand why it is beyond the scope of the study to identify upstream regulators and downstream targets, especially if we are talking about Nature Immunology as the target journal. Furthermore, the functional significance of the findings in vivo was not addressed either.
Finally, if a substantial fraction of the ILC1 examined might belong to ILC3, this has to be investigated in more depth.

If this is stochastic fate mapping in precursors could be easily checked by measuring fate mapping positive ILC2.

Do ILC3-ILC1 appear as a population distinct from ILC1 when analyzing the single-cell sequencing data?

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A31400B

Message: In reply please quote: NI-A31400B

Dear Georg,

I am delighted to accept your manuscript entitled "Effector differentiation downstream of lineage commitment in ILC1 is driven by Hobit across tissues" for publication in an upcoming issue of Nature Immunology.

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

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

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Kind regards,

Laurie

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