RESEARCH NOTE

Conventional NK cells and ILC1 are partially ablated in the livers of Ncr1iCreTbx21fl/fl mice [version 1; referees: 2 approved with reservations]

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Abstract

Mouse liver contains both Eomes-dependent conventional natural killer (cNK) cells and Tbet-dependent liver-resident type I innate lymphoid cells (ILC1). In order to better understand the role of ILC1, we attempted to generate mice that would lack liver ILC1, while retaining cNK, by conditional deletion of Tbet in NKp46+ cells. Here we report that the Ncr1iCreTbx21fl/fl mouse has a roughly equivalent reduction in both the cNK and ILC1 compartments of the liver, limiting its utility for investigating the relative contributions of these two cell types in disease models. We also describe the phenotype of these mice with respect to NK cells, ILC1 and NKp46+ ILC3 in the spleen and small intestine lamina propria.

Corresponding author: Victoria Male (v.male@ucl.ac.uk)

Author roles: Cuff AO: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; Male V: Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction

Mouse liver contains two NK cell populations. Conventional NK cells (cNK) are defined by their expression of CD49b (DX5), depend on the transcription factor Eomes, and circulate freely. The other NK cell population, which expresses CD49a, depends on the transcription factor Tbet and is unable to leave the liver. There is still some dispute over whether these cells should properly be considered liver-resident NK cells or non-NK type I innate lymphoid cells (ILC1). Here, we call them “liver ILC1.” In mice, cNK and liver ILC1 are distinct lineages that cannot cross-differentiate.

The factors involved in the lineage specification of liver ILC1 are already well-understood, but the function of these cells is not yet clear. They produce IFNγ and TNFα, as expected of ILC1, as well as high levels of GM-CSF, but it is unclear whether the production of these cytokines specifically by liver ILC1, as opposed to cNK, have any role in health and disease. Tissue-resident ILC1 in some other organs have physiological functions, so it is also possible that liver ILC1 have some as-yet-undiscovered physiological role.

To answer these questions, we sought to generate mice that would lack liver ILC1 while retaining cNK. Tbet knockout (Tbx21−/−) mice fulfill these criteria, but also have alterations in the T cell compartment that would complicate the analysis. Therefore, we crossed Tbx21−/− onto Ncr1+fl/fl mice to produce conditional knockout (Ncr1+fl/fl−/−) animals, in which Tbet is lost only in NKp46+ cells. Here, we report that these mice have a roughly equivalent reduction in both the cNK and ILC1 compartments of the liver, limiting their utility for investigating the relative contributions of these two cell types in disease models. We also note that the loss of Tbet differentially impacts NKp46+ ILC populations in the spleen, liver and small intestine, suggesting that Ncr1+fl/fl−/− mice could have potential as a tool for understanding how and when Tbet is required for NKp46+ ILC development and trafficking.

Materials and methods

Mice

B6(Cg)-Ncr1tm1.1(icre)Viv/+Orl mice (RRID MGI:5309017; “Ncr1+cre”) were acquired from the European Mutant Mouse Archive as frozen embryos and rederived in house. B6.129-Tbx21tm2Srnr/J mice (RRID IMSR_JAX:022741; “Tbx21+btn”) were acquired from the Jackson Laboratory. Ncr1+cre mice were crossed onto Tbx21+btn and the resultant F1 generation was backcrossed onto Tbx21+btn to produce Ncr1+cre Tbx21+btn conditional knockouts (n = 6) and Ncr1+cre Tbx21+btn littermate controls (n = 6). Mice were sacrificed between 6.5 and 9 weeks of age, using rising carbon dioxide followed by cervical dislocation. Animal husbandry and experimental procedures were performed according to UK Home Office regulations and institute guidelines, under project license 70/8530.

Cell isolation

Dissected livers (a total of 12) were minced finely with opposing scalpel blades. The tissue was collected in HBSS with Ca2+ and Mg2+ (Life Technologies, Paisley, UK) supplemented with 0.01% collagenase IV (Life Technologies) and 0.001% DNase I (Roche, distributed by Sigma-Aldrich, Dorset, UK) and passed through a 70 μm cell strainer. The suspension was spun down (500 g, 4°C, 10 minutes) and the pellet resuspended in RPMI 1640 medium (Life Technologies). The cell suspension was then layered over 24% Optiprep (Sigma-Aldrich) and centrifuged without braking (700 g, RT, 20 minutes). The interface layer was taken and washed in HBSS without Ca2+ and Mg2+ (Lonz, distributed by WVR, Lutterworth, UK) supplemented with 0.25% bovine serum albumin (Sigma-Aldrich) and 0.001% DNase I.

Small intestine lamina propria lymphocytes were isolated using a protocol adapted from Halim and Takei. Briefly, dissected intestines (a total of 12) were placed in ice-cold PBS supplemented with 2% fetal calf serum (FCS; Life Technologies) and the bulk of fecal matter removed by flushing the intestines using a syringe and 18G blunt end needle. The intestines were cut longitudinally and vortexed briefly 3x in ice-cold PBS/2% FCS to remove residual fecal matter. Tissue sections were incubated in PBS supplemented with 1 nM EDTA (shaking at 120 rpm, 37°C, 20 minutes) followed by 3x washes with ice-cold PBS/2% FCS before being minced finely with opposing scalpel blades. The homogenized tissue was digested in DMEM (Life Technologies) supplemented with 10% FCS, 50 μM 2-mercaptoethanol (Life Technologies), 250 U/mL collagenase IV and 50 U/mL DNase I (shaking at 120 rpm, 37°C, 20 minutes) and passed through a 70μm cell strainer. The cell suspension was centrifuged (400 g, 4°C, 5 minutes) and the cell pellet resuspended in 40% Percoll (GE Healthcare, distributed by Sigma-Aldrich) before centrifugation without braking (600 × g, 4°C, 10 minutes). The resultant pellet was washed in PBS/2% FCS (400 × g, 4°C, 5 minutes).

Dissected spleens (a total of 12) were passed through a 40 μm cell strainer. Red blood cells were lysed by 5 minute incubation in ACK lysing buffer (Life Technologies).

Flow cytometry

The antibodies used are displayed in Table 1.

The lineage cocktail consisted of CD3, CD8α, CD19 and Gr1 (Biolegend, London, UK). Dead cells were excluded using fixable viability dye eFluor 450 (eBioscience, San Diego, CA, USA) (4°C, 15 minutes). Surface staining was carried out in PBS supplemented with 1% FCS (4°C, 15 minutes). Intracellular staining was carried out using Human FoxP3 Buffer (BD Biosciences, Oxford, UK), according to the manufacturer’s instructions. Data were acquired on an LSRFortessa II (BD Biosciences) and analyzed using FlowJo v.X.0.7 (RRID SCR_008520; Tree Star, Ashland, OR, USA).
Table 1. Antibodies used for flow cytometry analysis.

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Statistical analysis
Groups were compared using Mann-Whitney U Tests. Analysis was carried out using Vassarstats (RRID SCR_010263).

Results
See Figure 1.

Discussion
We observed a modest (~4-fold) reduction in splenic NK in the Ncr1iCre Tbx21fl/fl conditional knockouts, compared to Ncr1WT Tbx21fl/fl littermate controls (Figures 1A and B). This is comparable to the 2- to 4-fold reduction in splenic cNK that has previously been reported in Tbx21 knockout, compared to wild-type, mice2,3,11 and is likely to be a result of reduced survival11 or bone marrow egress12 of NK cells in the absence of Tbet.

We also observed a reduction in the absolute number of cNK in the liver (Figures 1C and D). We had expected that this might be similar to the reduction of cNK in the spleen, but, at ~10-fold, it was more pronounced, potentially pointing towards a differential requirement for Tbet in cNK survival in or recruitment to the liver, compared to the spleen. Although the absolute number of liver ILC1 was also reduced (~10-fold), a substantial residual population was present (Figures 1C and E), in contrast to the Tbx21 knockout, in which these cells are almost completely eliminated3. Unlike the cNK in the conditional knockout, the residual liver ILC1 all expressed Tbet (Figure 1C). This supports the proposal that Tbet is absolutely required for continued survival of these cells, since no ILC1 in which Tbet was not expressed persisted. The failure to completely excise Tbx21 in ILC1 could be consistent with a recent report that Ncr1iCre expression is less penetrant in these cells than in cNK3,13, or the excision could have failed in only a few cells that then benefited from a selection advantage. Whatever the cause of the unexpectedly large reduction in cNK and the unexpectedly small reduction in ILC1, the finding that both of these were reduced by equivalent amounts in the liver of conditional knockouts compared to controls limits the utility of Ncr1iCre Tbx21fl/fl mice for dissecting the relative contributions of the two cell types in disease models.

Rankin et al. have also recently generated Ncr1iCre Tbx21fl/fl mice, and report a severe reduction in ILC1 and Nkp46+ ILC3 in the small intestine lamina propria14. We confirmed these findings in our conditional knockout mice, observing a ~3-fold reduction in ILC1 and a ~15-fold reduction in Nkp46+ ILC3 in the small intestine, compared to littermate controls (Figures 1F–H).

In summary, conditional deletion of Tbet in Nkp46+ cells, where Tbx21 excision has been successful, differentially affects cNK and ILC1 in different organs. In the liver, a residual population of ILC1, in which Tbx21 has not been excised, persists. We conclude that the Ncr1iCre Tbx21fl/fl mouse is therefore unlikely to be useful for...
Figure 1. Characterisation of innate lymphoid cell (ILC) populations in Ncr1<sup>iCre</sup>Tbx21<sup>fl/fl</sup> mice. Representative flow cytometry of leukocytes isolated from the (A) spleen, (C) liver and (F) small intestine lamina propria (siLP) of Ncr1<sup>WT</sup>Tbx21<sup>fl/fl</sup> (“control”) and Ncr1<sup>iCre</sup>Tbx21<sup>fl/fl</sup> (“cKO”) mice. Gated by scatter and on live, CD45+ cells. Summary data for cell frequency and absolute number in (B) spleen, (D,E) liver and (G,H) siLP. Each point represents a single mouse (n = 6), p values were determined using a Mann-Whitney U Test.
investigating the relative contributions of liver cNK and ILC1 to pathogenesis in disease models, but could still have potential as a tool for understanding how and when Tbet is required for the development and trafficking of NKp46⁺ ILC.

Data availability
Data is available at DOI, 10.17605/OSF.IO/GDMW.

Competing interests
No competing interests were disclosed.

Grant information
The study was funded by the Wellcome Trust [105677] (Royal Society and Wellcome Trust Sir Henry Dale Fellowship).

References

Open Peer Review

Current Referee Status: ? ?

Version 1

Referee Report 20 June 2017
doi:10.21956/wellcomeopenres.12683.r23309

Gabrielle T. Belz
Walter and Eliza Hall Institute of Medical Research, Melbourne, Vic, Australia

This is an interesting small study in which the objective was to delete Tbet and hopefully generate mice that lacked ILC1. This turned out not to be the case.

This is a very brief report that depends solely on the results shown in Figure 1. These data appear to support the claims of the authors that loss of T-bet is not sufficient to differentially delete ILC1. It is indicated that 6 mice have been used, however, it is not clear whether this represents a single experiment, or alternately, the data are pooled from several experiments. It is essential to clarify this point.

The authors have used NcrWTTbx21fl/fl control mice for their experiments. Ncr1iCre mice have lower levels of expression of NKp46 compared with either wildtype or floxed control mice. Given only floxed control mice are indicated to have been used, how was this reduction in expression accommodated, or was this considered in the experiments? It is not clear what gating strategy was used for enumerating the NK cell frequency or number.

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.
**Referee Expertise:** Immunology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**Author Response 03 Jul 2017**

**Victoria Male**, Royal Free and UCL School of Medicine, UK

This is an interesting small study in which the objective was to delete Tbet and hopefully generate mice that lacked ILC1. This turned out not to be the case.

This is a very brief report that depends solely on the results shown in Figure 1. These data appear to support the claims of the authors that loss of T-bet is not sufficient to differentially delete ILC1. It is indicated that 6 mice have been used, however, it is not clear whether this represents a single experiment, or alternately, the data are pooled from several experiments. It is essential to clarify this point.

Six littermate pairs (a total of 12 mice) were used, with spleen, liver and small intestine taken from each mouse. We have clarified this point in the Methods of version 2:

> “Ncr1 \textsuperscript{iCre} mice were crossed onto Tbx21 \textsuperscript{fl/fl} and the resultant F1 generation was backcrossed onto Tbx21 \textsuperscript{fl/fl} to produce Ncr1 \textsuperscript{iCre} Tbx21 \textsuperscript{fl/fl} conditional knockouts (n = 6) and Ncr1 \textsuperscript{WT} Tbx21 \textsuperscript{fl/fl} littermate controls (n = 6)... Spleen, liver and intestines were dissected out of each of the 12 mice for cell isolation.”

The authors have used NcrWTTbx21fl/fl control mice for their experiments. Ncr1iCre mice have lower levels of expression of NKp46 compared with either wildtype or floxed control mice. Given only floxed control mice are indicated to have been used, how was this reduction in expression accommodated, or was this considered in the experiments? It is not clear what gating strategy was used for enumerating the NK cell frequency or number.

In version 2, we discuss the reported phenotype of Ncr1 \textsuperscript{iCre} mice and the steps we have taken to avoid the potential confounding effect of slightly lower NKp46 expression in NKp46+ cells in these mice; in particular, that we identify NK cells using NK1.1, rather than Lin-NKp46+, to avoid potential confounding effects of reduced NKp46 expression.

> “Although we chose to use floxed-only, rather than iCre-only, littermate controls, we do recognise that iCre transgene expression itself can have an effect on phenotype. Ncr1 \textsuperscript{iCre} mice are known to have slightly reduced expression of NKp46 on NK cells, although the total number of NK cells (identified as CD3- NKp46+) in these mice is normal \textsuperscript{9}. We confirmed these observations in Ncr1 \textsuperscript{iCre} mice, compared to Ncr1 \textsuperscript{WT} littermate controls in our own colony \textsuperscript{15}. Further, we identify NK cells as Lin- NK1.1+, rather than Lin-NKp46+, to avoid potential confounding effects of reduced NKp46 expression.”

Gating strategies are as shown in the figure. For greater clarity, in version 2 we also give
the gating strategies used to define each population in the text.

**Competing Interests:** No competing interests were disclosed.

Referee Report 08 June 2017

doi:10.21956/wellcomeopenres.12683.r23310

David R. Withers  
Institute of Immunology and Immunotherapy, Institute of Biomedical Research, University of Birmingham, Birmingham, UK

The manuscript of Cuff and Male provides useful insight into the effects of deleting tbx21 in cells expressing NK1.1, resulting in loss of T-bet expression in a subset of immune cells. In the current climate of developing our understanding of mechanisms using conditional KO mice, basic data on how models work has clear value to the immunological community and data indicating the success of problems with this type of model can really benefit other researchers. It is also important for researchers to fully assess how well different conditional KO mouse models work with robust controls – it is now apparent that one cannot simply assume either specific or efficient cre-mediated deletion simply because of the intentioned design of the mouse model. Within the data presented here, it is striking that tissue specific effects are observed, particularly given the interest in tissue residency of ILC populations and the potential for tissue-specific differentiation. Much of the data is clearly presented and appears robust in terms of the analysis of NK/ILC1 populations. There are some modest concerns with manuscript as it stands - some in terms of presentation of the data and some in terms of the experimental setup and methodology.

**Experimental design:**
- The control mice used here are ‘floxed only’ controls rather than ‘cre only’ controls. The latter is better as it enables the impact of expression of the bacteriophage protein (cre), which is clearly a ‘foreign’ element expressed in the mouse. A number of studies have demonstrated that cre expression can impact on aspects of the immune system (e.g. Lck in T cell development in the thymus). This should be acknowledged within the description of the data.
- The authors should have fate-mapped cre expression in their mice in their hands to enable the efficiency of cre-expression to be properly addressed. This would clarify the extent to which NK1.1 expressing cells show evidence of cre mediated deletion, informing the understanding of NK1.1+ cells that retain T-bet expression. Whilst beyond the scope of this study now, it again could be noted.

**Methodology:**
- The SI LP prep is clearly sub-optimal for ILC populations – the fraction of the Lineage negative cells expressing IL-7Rα is substantially less than seen with other methods and there is clearly a substantial range in terms of numbers of cells isolated. Thus the data from the SI LP prep is of limited value given the spread of data and the obvious issues in cell isolation. Thus this data appears very preliminary and suboptimal - this should be recognised in the description of this data.

**Presentation of data:**
- In Figures B,D, E, G and H it would be more appropriate to show the median rather than the mean (which is what I assume the bar represents – it is not described in the figure legend). One cannot
assume the data is normally distributed and plotting the median would eliminate bias driven by clear outliers - for example the data in Figure H, absolute number clearly suffers from this, as does the similar data in ‘G’. The data in all these graphs should show median, not mean and this should be articulated in the Figure Legend.

- It is not clear to me in the SI LP analysis (in part ‘F’) why Lin-IL-7Ra+ cells are not gated on – there is a clear IL-7Ra+ population, but the gate includes IL-7Ra- cells. Given that the aim of this analysis is to look at ILC populations (but with no reference to conventional NK cells which are IL-7Ra-), a more specific gate would seem sensible – this would be my recommendation.

- Also in part F, the plots of T-bet expression would benefit from an indication of where the authors consider +ve and –ve T-bet staining. This would seem reasonably uncontroversial given the populations observed.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 03 Jul 2017

Victoria Male, Royal Free and UCL School of Medicine, UK

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In version 2, we acknowledge that Cre expression itself can have a phenotypic effect. We
discuss the reported phenotype of Ncr1 \textsuperscript{iCre} mice and the steps we have taken in this study to avoid the potential confounding effect of slightly lower Nkp46 expression in Nkp46+ cells in these mice, in particular that we identify NK cells using NK1.1, rather than Nkp46. We have also made the data from our own characterisation of Ncr1 \textsuperscript{iCre} compared to WT littermate control mice available, linked to this publication. In “Mice”:

“Although we chose to use floxed-only, rather than iCre-only, littermate controls, we do recognise that iCre transgene expression itself can have an effect on phenotype. Ncr1 \textsuperscript{iCre} mice are known to have slightly reduced expression of Nkp46 on NK cells, although the total number of NK cells (identified as CD3- Nkp46+) in these mice is normal \cite{9}. We confirmed these observations in Ncr1 \textsuperscript{iCre} mice, compared to Ncr1 WT littermate controls in our own colony \cite{15}. Further, we identify NK cells as Lin- NK1.1+, rather than Lin-, to avoid potential confounding effects of reduced Nkp46 expression.”

The authors should have fate-mapped cre expression in their mice in their hands to enable the efficiency of cre-expression to be properly addressed. This would clarify the extent to which NK1.1 expressing cells show evidence of cre mediated deletion, informing the understanding of NK1.1+ cells that retain T-bet expression. Whilst beyond the scope of this study now, it again could be noted.

Although we have not done this ourselves, fate mapping of the Ncr1 \textsuperscript{iCre} mice was done as part of the characterisation when they were first developed. We have added this to the Discussion of version 2:

“We were surprised to note that Tbx21 excision seemed to be less efficient in liver ILC1 than cNK, because fate mapping of iCre activity under the Ncr1 promoter using R26R\textsuperscript{eYFP} has previously shown that iCre activity is higher in ILC1 than cNK \cite{9}.”

The SI LP prep is clearly sub-optimal for ILC populations – the fraction of the Lineage negative cells expressing IL-7Rα is substantially less than seen with other methods and there is clearly a substantial range in terms of numbers of cells isolated. Thus the data from the SI LP prep is of limited value given the spread of data and the obvious issues in cell isolation. Thus this data appears very preliminary and suboptimal - this should be recognised in the description of this data.

We acknowledge this in version 2. In the Discussion:

“We were able to isolate fewer Lin- CD127+ ILC from the small intestine than has previously been reported, but even with this sub-optimal cell isolation procedure we made findings similar to that of Rankin \textit{et al}., observing a ~6-fold reduction in ILC1 (defined here as Lin- CD127+ RORγt+ Nkp46-) and a ~24-fold reduction in Nkp46+ ILC3 (defined here as Lin- CD127+ RORγt+ Nkp46+) compared to littermate controls (Figures 1F–H).”

In Figures B,D, E, G and H it would be more appropriate to show the median rather than the mean (which is what I assume the bar represents – it is not described in the figure legend). One cannot assume the data is normally distributed and plotting the median would eliminate bias driven by clear outliers - for example the data in Figure H, absolute number clearly suffers from this, as does the similar data in ‘G’. The data in all these graphs should show median, not mean and this should be articulated in the Figure Legend.
We have done this in version 2.

It is not clear to me in the SI LP analysis (in part ‘F’) why Lin-IL-7Ra+ cells are not gated on – there is a clear IL-7Ra+ population, but the gate includes IL-7Ra- cells. Given that the aim of this analysis is to look at ILC populations (but with no reference to conventional NK cells which are IL-7Ra-), a more specific gate would seem sensible – this would be my recommendation.

Because our aim in the siLP analysis was to confirm the findings from Rankin et al, we used their gating strategy: ILC1 and NKp46+ ILC3 as a proportion of total lineage negative cells. However, we do agree that gating on Lin- CD127+ cells is more appropriate for identifying ILCs and we have reanalysed the data using this strategy in version 2. Readers who wish to compare our data directly to that of Rankin et al may refer to version 1, which will still be available.

**Competing Interests:** No competing interests were disclosed.