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RESEARCH NOTE

REVISED Conventional NK cells and ILC1 are partially ablated in the livers of Ncr1^{iCre}Tbx21$^{fl/fl}$ mice [version 2; peer review: 2 approved]

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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 Ncr1^{iCre}Tbx21$^{fl/fl}$ 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.

Keywords

NK, liver, ILC1, ILC3, NKp46, Tbet, Ncr1, Tbx21

Open Peer Review

Reviewer Status ✓ ✓

	Invited Reviewers	
	1	**2**
REVISED **version 2** published 05 Jul 2017	✓ report	✓ report
version 1 published 07 Jun 2017	? report	? report

1 **David R. Withers**, University of Birmingham, Birmingham, UK

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

Any reports and responses or comments on the article can be found at the end of the article.

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

Competing interests: No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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First published: 07 Jun 2017, **2**:39 (https://doi.org/10.12688/wellcomeopenres.11741.1)

REVISED **Amendments from Version 1**

Version 2 contains the following changes:

- In Table 1, we give the dilution of NK1.1 antibody as 1/200, not 1/100 as was erroneously stated in version 1.
- In "Mice", we discuss the phenotype of the Ncr1-iCre mouse and the steps we have taken to avoid the potential confounding effect of the slightly lower expression of NKp46 that is known to occur in these mice.
- In "Discussion", we discuss the previously published data of the fate mapping on Ncr1-iCre mice.
- In "Discussion", we acknowledge the limitations of our siLP leukocyte isolation technique.
- In Figure 1, we reanalyse the siLP ILC populations using a Lin- CD127+ gating strategy, not Lin- only, as in version 1. Summary graphs now show medians, rather than means, as in version 1.

See referee reports

Introduction

Mouse liver contains two NK cell populations. Conventional NK cells (cNK) are defined by their expression of CD49b (DX5)[1], depend on the transcription factor Eomes[2], and circulate freely[1,3]. The other NK cell population, which expresses CD49a[1], depends on the transcription factor Tbet[2,3] and is unable to leave the liver[1,3]. 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)[4,5]: here, we call them "liver ILC1". In mice, cNK and liver ILC1 are distinct lineages that cannot cross-differentiate[6].

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[1–3,6], but it is unclear whether the production of these cytokines specifically by liver ILC1, as opposed to by cNK, have any role in health and disease. Tissue-resident ILC1 in some other organs have physiological functions[7,8], 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[2,3], but also have alterations in the T cell compartment that would complicate the analysis. Therefore, we crossed $Tbx21^{fl/fl}$ onto $Ncr1^{iCre}$ mice to produce conditional knockout ($Ncr1^{iCre}$ $Tbx21^{fl/fl}$) animals, in which Tbet is lost only in cells expressing Ncr1, whose protein product is the NK cell activating receptor NKp46. 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 $Ncr1^{iCre}$ $Tbx21^{fl/fl}$ 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)-$Ncr1^{tm1.1(icre)Viv}$/Orl mice[9] (RRID MGI:5309017; "$Ncr1^{iCre}$") were acquired from the European Mutant Mouse Archive as frozen embryos and rederived in house. B6.129-*Tbx21*^{tm2Srnr}/J mice (RRID IMSR_JAX:022741; "$Tbx21^{fl/fl}$") were acquired from the Jackson Laboratory. $Ncr1^{iCre}$ mice were crossed onto $Tbx21^{fl/fl}$ and the resultant F1 generation was backcrossed onto $Tbx21^{fl/fl}$ to produce $Ncr1^{iCre}$ $Tbx21^{fl/fl}$ conditional knockouts (n = 6) and $Ncr1^{WT}$ $Tbx21^{fl/fl}$ littermate controls (n = 6).

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. $Ncr1^{iCre}$ 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[9]. We confirmed these observations in $Ncr1^{iCre}$ mice, compared to $Ncr1^{WT}$ littermate controls in our own colony[10]. Further, we identify NK cells as Lin- NK1.1+, rather than Lin- NKp46+, to avoid potential confounding effects of reduced NKp46 expression.

Mice were sacrificed between 6.5 and 9 weeks of age, using rising carbon dioxide followed by cervical dislocation. Spleen, liver and intestines were dissected out of each of the 12 mice for cell isolation. 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 Ca^{2+} Mg^{2+} (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 Ca^{2+} Mg^{2+} (Lonza, distributed by VWR, 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[11]. 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).

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 (defined as Lin- NK1.1+) in the $Ncr1^{iCre}$ $Tbx21^{fl/fl}$ conditional knockouts, compared to $Ncr1^{WT}$ $Tbx21^{fl/fl}$ 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, mice[2,3,12] and is likely to be a result of reduced survival[12] or bone marrow egress[13] of NK cells in the absence of Tbet.

We also observed a reduction in the absolute number of cNK (defined as Lin- NK1.1+ CD49b+) 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 (defined as Lin- NK1.1+ CD49a+) 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 eliminated[2,3]. 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[2], since no ILC1 in which Tbet was not expressed persisted. We were surprised to note that Tbx21 excision seemed to be less efficient in liver ILC1

Table 1. Antibodies used for flow cytometry analysis.

Antibody	Clonality	Fluorophore	Dilution	Host Animal	Manufacturer	Catalog #	RRID
CD3	17A2	FITC	1/200	Rat	Biolegend	100203	AB_312660
CD8α	53-6.7	FITC	1/200	Rat	Biolegend	100705	AB_312744
CD19	6D5	FITC	1/200	Rat	Biolegend	115505	AB_313640
Gr1	RB6-8C5	FITC	1/200	Rat	Biolegend	108405	AB_313370
NK1.1	PK136	APC-eFluor 780	1/200	Mouse	eBioscience	47-5941	AB_2637449
CD45	30-F11	Brilliant Violet 510	1/200	Rat	Biolegend	103137	AB_2561392
CD49a	Ha31/8	Alexa Fluor 647	1/100	Hamster	BD Biosciences	562113	AB_11153312
CD49b	DX5	PerCP-eFluor 710	1/200	Rat	eBioscience	46-5971	AB_11149865
CD127	A7R34	PE	1/100	Rat	eBioscience	17-1271	AB_469435
NKp46	29A1.4	PerCP-eFluor 710	1/50	Rat	eBioscience	46-3351	AB_1834441
Eomes	Dan11mag	PE-eFluor 610	1/100	Rat	eBioscience	61-4875	AB_2574614
Eomes	Dan11mag	PE-Cyanine7	1/100	Rat	eBioscience	25-4875	AB_2573453
Tbet	eBio4B10	eFluor 660	1/100	Mouse	eBioscience	50-5825	AB_10596655
Tbet	eBio4B10	PE-Cyanine7	1/100	Mouse	eBioscience	25-5825	AB_11041809
RORγt	Q31-378	PE-CF594	1/100	Mouse	BD Biosciences	562684	AB_2651150

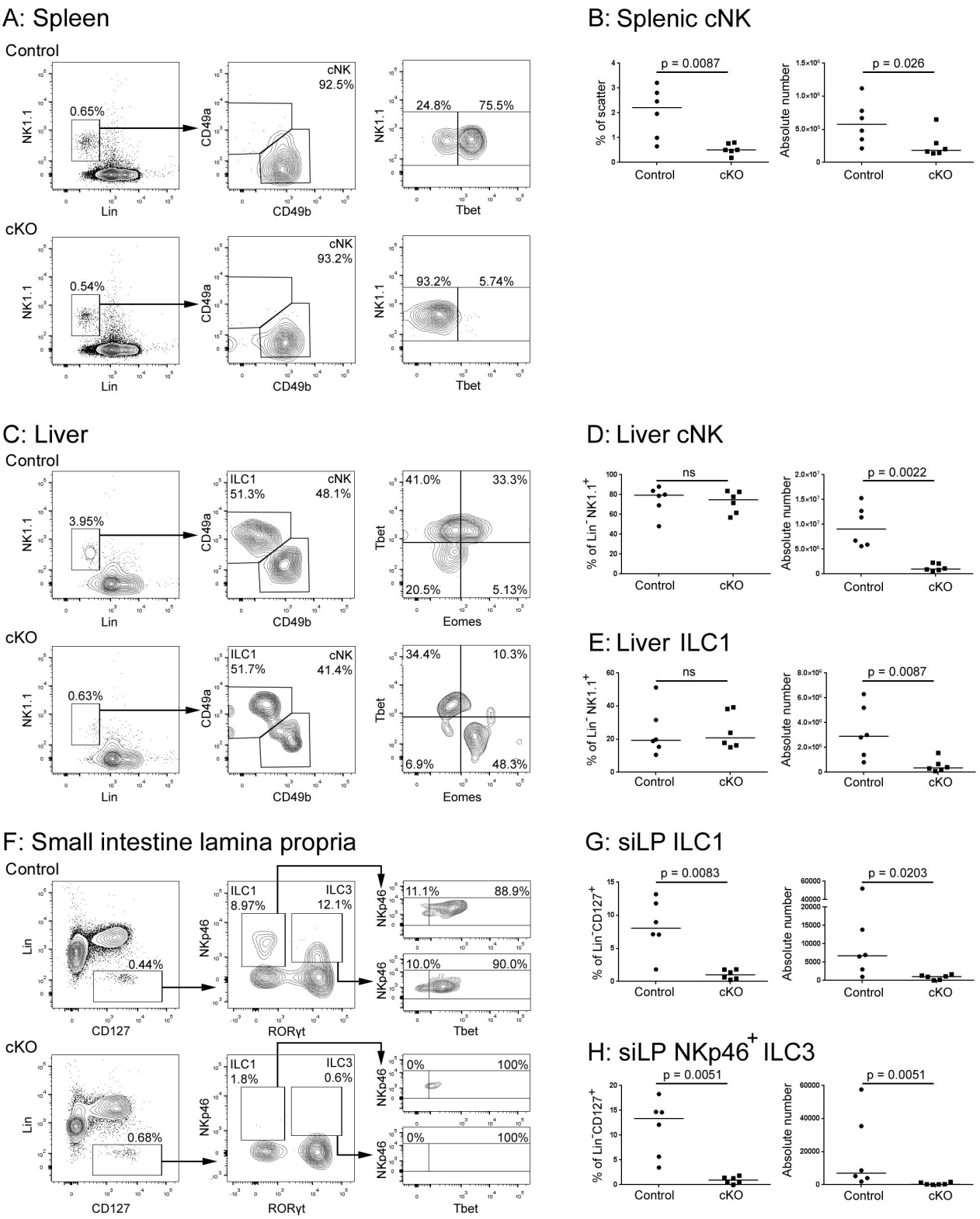

Figure 1. Characterisation of innate lymphoid cell (ILC) populations in Ncr1^{iCre}Tbx21$^{fl/fl}$ mice. Representative flow cytometry of leukocytes isolated from the (**A**) spleen, (**C**) liver and (**F**) small intestine lamina propria (siLP) of Ncr1^{WT} Tbx21$^{fl/fl}$ ("control") and Ncr1^{iCre} Tbx21$^{fl/fl}$ ("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 data from a single mouse (n = 6 per group), bars represent the medians and p values were determined using a Mann-Whitney U Test.

than cNK, because fate mapping of iCre activity under the Ncr1 promoter using $R26R^{eYFP}$ has previously shown that iCre activity is higher in ILC1 than cNK[9]. Given the absolute requirement of Tbet for the survival of liver ILC1[2,3], it seems likely that even if the excision failed in only a few cells, these would have benefited from a selection advantage that might have resulted in their expansion. 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 $Ncr1^{iCre}$ $Tbx21^{fl/fl}$ mice for dissecting the relative contributions of the two cell types in disease models.

Rankin *et al.* have also recently generated $Ncr1^{iCre}$ $Tbx21^{fl/fl}$ mice, and report a severe reduction in ILC1 (defined in their paper as Lin- RORγt+ NKp46-) and $NKp46^+$ ILC3 (defined as Lin- RORγt+ NKp46+) in the small intestine lamina propria[14]. We were able to isolate fewer Lin- CD127+ ILC from the small intestine than has previously been reported, but even with this suboptimal cell isolation procedure we made findings similar to those of Rankin *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 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 $Ncr1^{iCre}$ $Tbx21^{fl/fl}$ mouse is therefore unlikely to be useful for 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/GDMWT[10].

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).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Version 2

Reviewer Report 28 July 2017

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

I have no further comments to make, I approve the manuscript.

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.

Reviewer Report 10 July 2017

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

This manuscript is much improved and provides a relevant contribution to the field.

It would be helpful to extend the discussion on the relevance of this work to already published studies. For example, Pikovskaya et al. (2016)[1]. This study also indicates that T-bet is inefficiently deleted.

It remains unclear why NCRiCre controls are not reported in this study as they appear to have been analysed. Nevertheless the authors have added a comment to the rationale of their exclusion from the dataset.

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Competing Interests: No competing interests were disclosed.

Reviewer 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.

Version 1

Reviewer Report 20 June 2017

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?

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.

Reviewer 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.

Author Response 03 Jul 2017

Victoria Male, UCL Medical School, London, 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 ^{iCre} mice were crossed onto Tbx21 $^{fl/fl}$ and the resultant F1 generation was backcrossed onto Tbx21 $^{fl/fl}$ to produce Ncr1 ^{iCre} Tbx21 $^{fl/fl}$ conditional knockouts (n = 6) and Ncr1 ^{WT} Tbx21 $^{fl/fl}$ 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 ^{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 NKp46. We have also made the data from our own characterisation of Ncr1 ^{iCre}

compared to WT littermate control mice available, linked to this publication.

"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 ^{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 [9]. We confirmed these observations in Ncr1 ^{iCre} mice, compared to Ncr1 ^{WT} littermate controls in our own colony [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.

Reviewer Report 08 June 2017

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?

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 uncontentious 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, UCL Medical School, London, UK

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.

In version 2, we acknowledge that Cre expression itself can have a phenotypic effect. We discuss the reported phenotype of Ncr1 ^{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 ^{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 ^{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 [9]. We confirmed these observations in Ncr1 ^{iCre} mice, compared to Ncr1 ^{WT} littermate controls in our own colony [15]. Further, we identify NK cells as Lin- NK1.1+, rather than Lin- NKp46+, 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 ^{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^{eYFP} has previously shown that iCre activity is higher in ILC1 than cNK [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 *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.