nature research

Peer Review Information

Journal: Nature Immnuology
Manuscript Title: RORα is a critical checkpoint for T cell and ILC2 commitment in the embryonic thymus
Corresponding author name(s): Ana C. F. Ferreira, Andrew N. J. McKenzie

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A29357

Message: 17th Apr 2020

Dear Andrew & Ana,

Thanks so much for providing your point-by-point response to the referees comments on your manuscript entitled "RORa expression is a critical checkpoint for the bifurcation of the T cell and ILC2 lineages in the embryonic thymus". 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 that addresses these serious concerns.

As noted in your response, a large majority of the comments can be addressed by adding already existing supplementary data and revising the text, there are several new experiments that are required and that you have noted that you can perform.

1. Additional replicate of genomic transcription factor binding and chromatin accessibility experiments. Q1 asked for this.

2. Colocalization of ILC2 and IL-33+ stromal cells. To address this question, we can intercross dual Bcl11b-tdTom, Id2-BFP mice with IL-33-citrine reporter mice, and analyse the fetal thymus by confocal microscopy. Q3 asked for this.

3. Do ILC2 inhibit T cell development. We will do this by incorporating congenically marked ILC2p into the Rora-deficient OP9-DL cultures, and determine if T cells still arise from the Rora-deficient ETPs. Q3 asked for this.

4. We will try to use retroviruses to introduce and overexpress Rora in ETP. We currently have protocols in the lab to allow us to transduce CLPs

and we will attempt to adapt this system for use with ETPs. Q3 asked for this.

5. In reference to Q2's point that a single-cell clonal analysis is needed to show that ETPs can give rise to ILC2 cells, you can cite the previous literature, including your 2012 NI study and a subsequent Wang 2017 report confirming those results). Thus, the single-cell clonal assay can be waived at this time.

Please keep us up to date with the accessibility of your laboratory and ability to be able to perform these new experiments in light of the current situation surrounding the SARS-CoV2 pandemic.

Please revise your manuscript taking 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.

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

Thank you for the opportunity to review your work.

Kind regards & stay well,

Laurie

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Referee expertise:

Referee #1: Transcriptional regulation immune cells

Referee #2: Innate immune cells

Referee #3: Innate immune cells

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors' discovery that RORa regulates a new pathway enabling ILC2 to branch off from T-cell development in the fetal thymus is exciting and persuasive. While most work on characterization of immature thymocytes has focused on the populations most enriched for T-lineage potential and T-program bias, which highly express c-Kit, in this manuscript the authors have looked at DN1 fetal thymocytes more broadly and have discovered an entirely separate lymphoid development track. Their data help to resolve how ILC2s can develop divergent properties from T lineage cells, becoming dependent on Id2 expression, despite sharing a dependence on GATA-3, TCF-1, and Bcl11b which can repress Id2 in T cells. The key mechanism that they focus on is the ability of Rora to activate Nfil3 and Id2 regardless of the presence of Bcl11b, and thus to override repression of Id2 by Bcl11b.

The ILC2-like population demonstrated in fetal mouse thymus is clearly distinct from the T-cell precursors and the NK precursors in a single-cell RNA-seq analysis, in agreement with hints from previous fetal mouse data (Kernfeld et al., 2018). Interestingly, human fetal thymus samples in single-cell RNA analysis have seemed to show a stronger population with an ILC3 signature (Zeng et al., 2019; Park et al, 2020), not found here. These ILC2s from the fetal mouse thymus are also shown to be able to seed the small intestine lamina propria and lung, although less efficiently than bone marrow-derived ILC2s. A particularly elegant feature of this paper is the use of mice with five different fluorescent reporters to track the expression patterns of the lineage-defining transcription factors in single cells: Id2, Bcl11b, Gata3, Rorc, and Rora. As a result, cell types are much better discriminated than would have been possible using cell surface markers alone, and likely activity balances are revealed between the exact regulatory genes that are best candidates to determine cell fate themselves. The authors (1) demonstrate the derivation of these Rora+ Id2+ Bcl11b+ Gata3-high ILC2s, as well as Rora- Id2+ Bcl11b Gata3-low NK cells, from ETP precursors, and also show that (2) IL-33 from Epcam-negative thymic stromal cells may be one of the inductive signals for their generation as well as a suppressive signal for the T-cell fate. The second point becomes important to demonstrate the importance of Rora, for data are shown that IL-33 cannot suppress T cell development in precursors from which Rora is conditionally deleted. Finally, the authors show very interesting preliminary results for the binding patterns of Rora and Gata3 on critical genomic loci, which support a new gene network model, as discussed in specific points. These findings together constitute an exciting advance, although some links to other work could be strengthened.

1. It is unfortunate that in the manuscript as submitted, it appears that all the genomic transcription factor binding and chromatin accessibility results were from single replicates. In principle, this part of the study could be a strength of the paper. The authors use a very creative approach to bypass a lack of appropriate ChIP-seq antibodies, and this will be of wide interest in itself. The results are intriguing, with support from motif analysis, and they suggest many notable details about regulation of important genes. If confirmed, these results would be most valuable. However, these results need to be replicated before they can be interpreted definitively. Replication of these results, and evaluating statistical support for their implications, is highest priority.

2. For researchers studying T cell development, c-Kit is a very important marker for subdividing progenitor populations. Fig. 2f does suggest that the ILC2 cells are not maximally c-Kit-high, at least not by the time they express Il13. However, the authors should include panels with c-Kit vs. CD44 and c-Kit vs. CD25 in the characterization of the starting thymocyte populations to show where the c-Kit levels of the ILC2 cells fall compared to classic T-cell precursors. These gates are also important to show how the ETP precursors were defined. If ETPs in this study were defined only as Id2-negative Bcl11b-negative DN1 cells, how does that overlap with the usual definition of ETPs based on c-Kit expression level in DN1 cells?

3. The comparison with implied regulatory pathways for the bone marrow cells is worth discussing in more depth. Here, Rora is suggested to be able to break the T-lineage affiliation of cells after they have entered the thymus, possibly after Bcl11b is already on.

a. Do the RNA expression data allow the estimated timing of Rora and Bcl11b expression in T-ILC2p to be compared with that in bone marrow-derived ILC2P lineages?

b. Is Zbtb16 expressed in any subset of the T-ILCp? What about Pdcd1? These are not shown in the heat maps, but are relevant to the question of whether the cells are differentiating locally through a consensus ILC2 pathway, or whether they are bypassing a ChILP-like stage.

4. The gene network model is exciting, but some of the edges could be strengthened with more direct evidence, if this is technically possible. Is there direct functional evidence for Rora acutely activating Nfil3 and Id2 expression? The binding of Rora to the Nfil3 and Id2 loci in Fig. 7 is intriguing but not proof that it is directly turning these genes on.

a. For example, if possible to examine the cells used in Fig. 6a,b at an earlier timepoint of culture +/- IL-33, it might be possible to test whether the reason T cell development proceeds in Rora KO cells in the presence of IL-33 might be that Id2 needs Rora to be upregulated.

b. Fig. 6a,b needs to show the total number of cells in these conditions, to assess the actual reduction in ILC2 cell number in Rora-KO cells.

Minor points

5. It would be very helpful if the paper could include Supplementary Tables with the actual measured levels of expression of genes from experiments shown in Fig. S1b, and the average cluster expression levels from Fig. 1c. This would not only make the results more informative, but also because levels matter. In Fig. 1c, for example, Rorc appears to be just as enriched in the ILC2 cluster as Rora (suggesting ILC3 development). Later evidence in the paper shows that this gives a misleading impression of its actual expression.

6. There is something strange in Fig. 1c with a gene, Bexp6. This is indicated as being enriched in two mutually exclusive patterns of expression, and the gene name itself seems to be a typo.

7. Slight overstatement on p. 7, line 16: the Il13 expression result shows that some ICOS+ cells and not ICOS- cells express, but it does not show that [all] ICOS+ cells express as suggested by the current wording.

8. There are some presentation problems in Fig. 2. In Fig. 2h, the overlays are not transparent enough and become very hard to distinguish where they overlap. For example, does the “red” group have a tail of high-perforin-expressing cells, or not? Increasing transparency or changing the order of the overlays could help. Also, the results in Fig. 2j would be more compelling at a larger size.

9. At the bottom of p. 8, the tSNE plot (Fig. 3f) shows that subsets within the ETP population are not discretely separated, but it does not show that the ETP population is “homogeneous”.

10. At the bottom of p. 10, a more involved line of logic appears to have been compressed to the point where it is obscure. It is explained in the Discussion, but if the connection is mentioned at this point in the Results, it would be helpful to spell out that these cells and

the gamma delta cells both express Ccr9, which has been shown to important for siLP homing.

11. On p. 17, lines 9-10, this should be one sentence rather than two.

Reviewer #2:
Remarks to the Author:
In this manuscript Ferreira and colleagues address multiple important questions in ILC2 biology including the origin of ILC2 and the transcriptional mechanisms that lead to ILC2 lineage determination. There are many different points made in the manuscript, as outlined below, but the most critical of these (in the opinion of this reviewer) is that the transcription factor RORa plays a role in controlling the ILC2 versus T lymphocyte fate choice in the embryonic thymus by directly regulating expression of Nfil3 and Id2 in cells that would otherwise extinguish their expression due to high expression of Bcl11b. The authors do provide some evidence that is consistent with this model including binding of RORa to the Nfil3 and Id2 genes in ILC2 and increased T cell differentiation in vitro in cultures seeded with RORa-deficient ETPs after culture in IL-2 + IL-33. However, they fail to prove the point by showing that single progenitor cells with the option of T lymphocyte or ILC2 potential, chose the T cell fate in the absence of RORa. They need to test the frequency of ILC2 and T cell progenitors at the single cell level to rule out outgrowth of T cells in the absence of ILC2.

There is a lot of interesting data presented in this manuscript but it is put together in a way that does not flow coherently and none of the points are demonstrated as well as they should be. The authors make the following conclusions: 1) that ILC2 arise from ETP and DN2 cells in the fetal thymus (E15.5 and E19.5) and can be identified using 5X polychromILC reporter mice; 2) that thymic ILC2 expand in IL-33 and that stromal cells are the major embryonic thymic cell type making IL-33; 3) that thymic ILC2 preferentially populate the intestine lamina propria; and 4) that RORa is required to suppress the T cell fate from ETPs and 5) that RORa binds to DNA near Nfil3 and Id2 as well as at Th2 cytokine loci to promote ILC2 development.

Additional comments:

1) The authors identified and characterized sub populations of T and ILC2 progenitors in the embryonic thymus using two separate methods, however in the main text they do not sufficiently show that these populations are the same. Figure 1 uses RNA-seq to describe the transcriptome of the 4 subpopulations identified by surface marker expression and Figure 2 identifies 4 populations based on BC11b- and Id2-reporter expression. In the main figures they do not correlate the populations from Figure 1 and Figure 2, leaving some doubt about the legitimacy of an ILC2 population, identified via transcriptomics in figure 1, and the BC11b/Id2 double positive population, identified in figure 2, being the same subset of cells. The authors attempt to rectify this in the supplement, by comparing the transcriptomes of the populations identified in both figures. To confidently compare the populations, there should be an analysis showing the relative correlation of the transcriptomes of cell populations from both figures.

2) The authors use FTOC to characterize the 4 populations that they identify by Id2- and Bcl11b-reporter expression. They indicate that they were unable to perform single cell

experiments under these conditions and therefore only bulk populations are analyzed. To demonstrate a single cell origin for ILC2 and T cells they really should do single cell experiments, and this should be feasible in OP9-DL1 cultures (for example in Figure 6). However, the result is not unexpected given previous studies from the Sun lab, which are not referenced in this manuscript but should be (Qian L., et al., J Exp. Med. 2019 Apr 1:21(4):884-899. And Wang H, et al. J Immunol. 2017 Apr15:198(8):3149-3158.)

3) The points about thymic stromal IL-33 being sufficient to promote ILC2 development seem out of place in this manuscript. The authors show, using
IL-33 reporter mice that the major IL-33 producing cells in the embryonic thymus are EpCAM- thymic stromal cells. However, in the supplemental material, they show that IL-33 is not required for thymic ILC2 development. Thus, this data does not provide anything important for the paper or impactful for the development of ILC2 in the embryo.

4) The data showing that thymic ILC2 preferentially colonize the intestinal lamina propria is also not fully developed and does not contribute substantially the major points raised in this manuscript. The authors demonstrate this intestinal LP homing preference by transplanting fetal thymus under the kidney capsule of adult mice and therefore it is not clear whether their natural tropism during fetal life is being revealed. Moreover, the experiment of co-transplantation of fetal thymic and bone marrow derived ILCs does not show a major tissue tropism for thymic-derived ILC2 (they are present at about 20% of control in both the iLP and lung). These experiments have very different contexts and it is difficult to interpret how these tropisms might reflect normal migration patterns. In this respect, again the authors should have referenced Sun et al, who show that ILC2 fate-mapped using LckCre are present in bone marrow, lung, and blood but not detectably in the siLP suggesting that thymic derived ILC2 can seed these tissues. The point of the authors here might have been to show Ccr9 on ILC2 derived from the thymus.

Minor comments:
1) The ATAC-seq and ChIP-seq data are presented as overlaid data that is difficult to follow for individual parameters. It would be better to present these as individual tracks stacked on top of each other. (For example see Shih et al., Cell 165(5):1120-1133, 2016).
2) The ILC versus T cell fate decision has also been shown to involve the Id2-E protein axis as mentioned in this paper but there were no references given. Miyazaki M et al, Immunity 2017, 46(5):818-834 should have been mentioned and perhaps Xu W et al, Blood 2013, 121(9):1534-1542, which doesn't identify ILC2 but shows a good single cell analysis for T cell/ILC fate.

Reviewer #3:
Remarks to the Author:
Ferreira and colleagues study the role for RORa in the generation of T cells versus ILC2 from early T cell precursors (ETP) in the fetal thymus. ETP are heterogeneous and can give rise to T cells as well as NK cells and DC in vitro. By using recently developed transcription factor (TF) reporter mice (polychromILC) that can read out expression of key TFs (Bcl11b, Id2, RORa, Gata3, Rorg), the authors dissect DN1 cells in the fetal thymus and and show that this population contains ETP but also NK and ILC2 precursors based on differential expression of Bcl11b and Id2. They further show that Bcl11b-Id2- cells can give rise to Bcl11b+Id2+ ILC2P as well as Bcl11b-Id2+ NKp in fetal thymic organ culture. As IL-33 can expand mature ILC2, the authors study IL-33 expressing cells using an IL-33

reporter mouse strain and document IL-33+ stromal cells in the fetal thymus; they further show that adding IL-33 to FTOC increases expression of Id2, Gata3 and CD25 on ILC2P populations. The authors next graft fetal thymus from polychromILC mice to Rag2-/-/gc-/- recipients and detect progeny in several organs which include ILC2 primarily in the gut. They compare the capacity of ILC2P from bone marrow versus thymus to repopulate Rag2-/-/gc-/- recipients and show that both are competent although bone marrow cells are more efficient. Since the authors find that RORa is differentially between Bcl11b-Id2- ETP and Bcl11b+Id2+ ILC2P, they next attempt to dissect the role for RORa in ILC2 versus T cell development in the thymus. Using OP9 stromal based cultures the authors find that RORa-deficient ETP can generate T cells but not ILC2 (as expected) while addition of IL-33 to these cultures promotes ILC2 at the expense of T cells in control ETP but not RORa-deficient ETP. The authors conclude that RORa not only promotes ILC2 development but reciprocally represses T cell development. The authors next take advantage of T2A sequences in their RORa and Gata3 reporter constructs to study DNA binding sites of these TFs in mature ILC2. They use this dataset to compare with ATACseq accessibility profiles in fetal thymus ETP, ILC2P, NKp and DN2/3 cells to construct a TF interaction map between RORa, Gata3, Bcl11b, Id2 and Nfil3. Essentially, the model proposes that RORa competes with Bcl11b to regulate T cell versus ILC2 development. In the case of T cells, the absence of RORa allows Bcl11b to repress Id2/Nfil3-mediated ILC development, while in ILC2, RORa competes with Bcl11b thereby allowing Id2/Nfil3 expression at the expense of T cells.

This is an elegant work to dissect the complex TF networks that operate during early T cell and ILC development.

1) The authors carefully assess the molecular signatures of fetal thymus DN1 cells at different embryonic stages. The scRNAseq and analysis of polychromILC mice suggest four or five major subsets (depending on age) including ETP, ILC2P, NKp and gd T cell-like cells. These can be visualized in polychromILC reporter mice by their differential expression of Bcl11b and Id2. One subset (Bcl11b+Id2-) is not discussed although the authors have performed RNA sequencing on this population. These cells at E15.5 and E19.5 appear to have high Gata3 levels (Figure2) but lack RORa suggesting that they are T cells. A fifth population that are proposed as gd T cells (Bcl11loId2lo) are related to this Bcl11b+Id2- subset?

2) The authors describe the ILC2P and NKp as precursors although their molecular signature clearly shows evidence of a mature phenotype (cytokine expression and relevant cytokine receptors to activate these cells in tissues). Most of the ILC2P appear to co-express mature cell markers (Supp fig 4e). This opens up the possibility that a fraction of the cells identified in the fetal thymus are relatively mature, even at E15.5. While the authors provide some evidence that these ILC2P and NKp are derived in situ via ETP (FTOC Fig 3), it remains possible that some of these cells gain access to the thymus after developing at other sites. Fetal ILC2 waves have been reported that seed may tissues during the embryonic period. In addition, an immature Bcl11b+Id2+ subset in the bone marrow that lacks mature ILC2 markers (ie: negative for IL-5, IL-13, IL-33R…) has been described. Do cells with this phenotype exist in the fetal thymus? If the almost fully mature ILC2 in the fetal thymus are derived from ETP, one might expect to find a trajectory of cells based on the scRNA seq analysis.

3) The results in Figure 3 provide strong evidence that ETP can generate ILC2 and NK-like cells in FTOC. Still, the efficiency of this process is not known and if would be helpful if the

authors provide absolute numbers of T cells versus ILC2/NK cells that are generated. Also, was IL-33 added to these cultures? While it is unlikely that contamination by Bcl11b+Id2+ cells during the sorting of Bcl11b-Id2- cells can explain the results (especially after transfer of 20 Bcl11b-Id2- cells), it would be interesting to know the expansion capacity of sorted Bcl11b+Id2+ cells and Bcl11b-Id2+ cells in FTOC (Fig 3g, h). How many Bcl11b+Id2+ and Bcl11b-Id2+ cells were transferred in the FTOC and how many cells were recovered?

4) The authors study IL-33 expression in the thymus using reporter mice. While about 10-20,000 IL-33+ stromal cells are detected, the analysis of IL-33 KO thymus shows that the 1-2,000 mature ILC2 expressing ST2 are not decreased. It is not clear whether these IL-33+ stroma localize near ILC2 cells in the thymus. Also the authors should comment on why there are apparently 10-fold more cytokine producing cells compared to cytokine responsive cells which contrasts with other cytokine systems in the thymus (IL-7 for example).

5) The authors propose a model for TF control of T versus ILC2 development with a critical role for RORa. This is in part based on Figure 6 which analyzes OP9 cultures of WT and RORa-deficient ETP. The authors conclude that RORa not only promotes ILC2 development but reciprocally represses T cell development. There are several points that still need to be addressed. First, it is not clear what cells are being studied in this experiment. The authors use 'ETP' but it is not clear exactly what markers have been used to isolate ETP from WT and RORA-deficient thymus and whether similarly isolated ETP from polychromILC represent a pure population of Bcl11b-Id2- cells (or not). This is important because if ETP from WT mice contain even a few ILC2P then these cells can explain the result. Second, assuming that the ETP are not containing any ILC2P, then an alternative explanation for the results must be excluded. It remains possible that ILC2 expansion in the presence of IL-33 in WT cultures have a suppressive effect on T cell development from ETP. The large expansion of IL-33 stimulated ILC2 would block T cell development and this would not occur in RORa-deficient cultures since ILC2 do not expand (perhaps due to lack of IL-33R?). Finally, there is no direct evidence that RORa represses T cell fate. RORa-deficient ETP generate T cells to a similar extent as WT ETP : no evidence for de-repression by RORa. The authors would need to over-express RORa in ETP and show that this represses T cell development.

6) While the authors model is interesting, one major question remains: how does RORa compete out Bcl11b to allow ILC2 development? The site in Id2 where Bcl11b may repress expression (+40 Kb downstream) is not bound by RORa.

Minor comments

1) the confocal analysis of ILC2 'foci' requires quantification.

Author Rebuttal to Initial comments

We would like to thank the reviewers for their positive and helpful comments. Following their observations, we have undertaken substantial additional experiments and added several new figure sections to address the issues and improve the clarity of our manuscript. We now submit a revised and enhanced version of the manuscript for consideration. We believe the manuscript is now much improved and we hope the reviewers will agree and find it suitable for publication.

Reviewer #1

(Remarks to the Author)
The authors' discovery that RORa regulates a new pathway enabling ILC2 to branch off from T-cell development in the fetal thymus is exciting and persuasive. While most work on characterization of immature thymocytes has focused on the populations most enriched for T-lineage potential and T-program bias, which highly express c-Kit, in this manuscript the authors have looked at DN1 fetal thymocytes more broadly and have discovered an entirely separate lymphoid development track. Their data help to resolve how ILC2s can develop divergent properties from T lineage cells, becoming dependent on Id2 expression, despite sharing a dependence on GATA-3, TCF-1, and Bcl11b which can repress Id2 in T cells. The key mechanism that they focus on is the ability of Rora to activate Nfil3 and Id2 regardless of the presence of Bcl11b, and thus to override repression of Id2 by Bcl11b.

The ILC2-like population demonstrated in fetal mouse thymus is clearly distinct from the T-cell precursors and the NK precursors in a single-cell RNA-seq analysis, in agreement with hints from previous fetal mouse data (Kernfeld et al., 2018). Interestingly, human fetal thymus samples in single-cell RNA analysis have seemed to show a stronger population with an ILC3 signature (Zeng et al., 2019; Park et al, 2020), not found here. These ILC2s from the fetal mouse thymus are also shown to be able to seed the small intestine lamina propria and lung, although less efficiently than bone marrow-derived ILC2s. A particularly elegant feature of this paper is the use of mice with five different fluorescent reporters to track the expression patterns of the lineage-defining transcription factors in single cells: Id2, Bcl11b, Gata3, Rorc, and Rora. As a result, cell types are much better discriminated than would have been possible using cell surface markers alone, and likely activity
balances are revealed between the exact regulatory genes that are best candidates to determine cell fate themselves. The authors (1) demonstrate the derivation of these Rora+ Id2+ Bcl11b+ Gata3-high ILC2s, as well as Rora- Id2+ Bcl11b Gata3-low NK cells, from ETP precursors, and also show that (2) IL-33 from Epcam-negative thymic stromal cells may be one of the inductive signals for their generation as well as a suppressive signal for the T-cell fate. The second point becomes important to demonstrate the importance of Rora, for data are shown that IL-33 cannot suppress T cell development in precursors from which Rora is conditionally deleted. Finally, the authors show very interesting preliminary results for the binding patterns of Rora and Gata3 on critical genomic loci, which support a new gene network model, as discussed in specific points. These findings together constitute an exciting advance, although some links to other work could be strengthened.

1. It is unfortunate that in the manuscript as submitted, it appears that all the genomic transcription factor binding and chromatin accessibility results were from single replicates. In principle, this part of the study could be a strength of the paper. The authors use a very creative approach to bypass a lack of appropriate ChIP-seq antibodies, and this will be of wide interest in itself. The results are intriguing, with support from motif analysis, and they suggest many notable details about regulation of important genes. If confirmed, these results would be most valuable. However, these results need to be replicated before they can be interpreted definitively. Replication of these results, and evaluating statistical support for their implications, is highest priority.

We have now performed and added further experimental repeats to support our findings. We now have 3 repeats of the ChIPseq and the ATACseq experiments. These additional data have been incorporated into a new Figure 6h and i, and will be deposited in GEO.

2. For researchers studying T cell development, c-Kit is a very important marker for subdividing progenitor populations. Fig. 2f does suggest that the ILC2 cells are not maximally c-Kit-high, at least not by the time they express Il13. However, the authors should include panels with c-Kit vs. CD44 and c-Kit vs. CD25 in the characterization of the starting thymocyte populations to show where the c-Kit levels of the ILC2 cells fall compared to classic T-cell precursors. These gates are also important to show how the ETP precursors were defined. If ETPs in this study were defined only as Id2-negative Bcl11b-negative DN1 cells, how does that overlap with the usual definition of ETPs based on c-Kit expression level in DN1 cells?

We thank the reviewer for raising this point. We have now added new figure S1b that shows c-Kit expression in the context of both CD44 and CD25 at day e15.5.

10

3. The comparison with implied regulatory pathways for the bone marrow cells is worth discussing in more depth. Here, Rora is suggested to be able to break the T-lineage affiliation of cells after they have entered the thymus, possibly after Bcl11b is already on.

a. Do the RNA expression data allow the estimated timing of Rora and Bcl11b expression in T-ILC2p to be compared with that in bone marrow-derived ILC2P lineages?

This is an interesting point. We have now added text to the Discussion. Within the limitations of the techniques, our RNAseq and flow cytometry data cannot discriminate any differences in the onset of Bcl11b, RORa and Id2 expression. These results suggest that their induction occurs in parallel, not stepwise – or is simply too closely linked for us to determine. For example, Pseudotime was performed and did not indicate a progression in stages. We have also now added a new supplementary figure in which we analysed Bcl11b, RORa and Id2 daily from E13 to E19 flow cytometry and were again unable to separate the temporal induction of these TFs (see Figure S1a).

b. Is Zbtb16 expressed in any subset of the T-ILCp? What about Pdcd1? These are not shown in the heat maps, but are relevant to the question of whether the cells are differentiating locally through a consensus ILC2 pathway, or whether they are bypassing a ChILP-like stage.

We have now added an additional new figure to supplementary figure 1 to show Zbtb16 and Pdcd1 expression (from RNAseq expts) (Fig S1e). Zbtb16 message is expressed at low levels in ETP, but at comparably higher amounts in ILC2p at both E15 and E19. Similarly, we observe an increase in Pdcd1 expression by ILC2p as compared to ETP at E19. Our data do not support the presence of ChILP-like cells in the thymus and suggest that ETPs are the only common progenitor and that they rapidly differentiate into ILC2p, pro-T and NKp cells. Thus, ILC differentiation in the embryonic thymus appears quite distinct from the ILC commitment we have observed in the BM. We have added text to discuss these findings in the Discussion.

4. The gene network model is exciting, but some of the edges could be strengthened with more direct evidence, if this is technically possible. Is there direct functional evidence for Rora acutely activating Nfil3 and Id2 expression? The binding of Rora to the Nfil3 and Id2 loci in Fig. 7 is intriguing but not proof that it is directly turning these genes on.

The reviewer raises an interesting point, especially regarding the technical ability to perform such experiments. The mechanism(s) will most likely involve other DNA binding factors and chromatin changes that cannot be addressed in a simple system. We have considered making mouse models to delete specific regions, but this would be extremely long-term and very costly. In vitro studies would be useful, but this would require studying gene expression in Rora-deficient progenitor cells during ILC development, but in the absence of Rora, ILC2 fail to develop. Thus, we cannot produce physiologically relevant cells for the study.

a. For example, if possible to examine the cells used in Fig. 6a,b at an earlier timepoint of culture +/- IL-33, it might be possible to test whether the reason T cell development proceeds in Rora KO cells in the presence of IL-33 might be that Id2 needs Rora to be upregulated.

We actually observe concomitant expression of both Id2 and Rora. We did try to address this question using the conditional deletion of RORa in Id2-BFP reporter mice. However, the tamoxifen treatment had non-specific effects on embryo development and the experiment had to be terminated.

b. Fig. 6a,b needs to show the total number of cells in these conditions, to assess the actual reduction in ILC2 cell number in Rora-KO cells.

As requested we have added 3 new graphs to Figure 6b and c to show the number of T cells and ILC2 in addition to the frequencies.

Minor points

5. It would be very helpful if the paper could include Supplementary Tables with the actual measured levels of expression of genes from experiments shown in Fig. S1b, and the average cluster expression levels from Fig. 1c. This would not only make the results more informative, but also because levels matter. In Fig. 1c, for example,

Rorc appears to be just as enriched in the ILC2 cluster as Rora (suggesting ILC3 development). Later evidence in the paper shows that this gives a misleading impression of its actual expression.

We have now added a new Table 1 to Supplementary data.

6. There is something strange in Fig. 1c with a gene, Bexp6. This is indicated as being enriched in two mutually exclusive patterns of expression, and the gene name itself seems to be a typo.

We are grateful to the reviewer for spotting this error, which we have now corrected.

7. Slight overstatement on p. 7, line 16: the Il13 expression result shows that some ICOS+ cells and not ICOS- cells express, but it does not show that [all] ICOS+ cells express as suggested by the current wording.

Thank you, yes, this is actually an important point and shows that there is heterogeneity in Il13 expression in ILC2 during this phase of ILC2p development in the thymus. We have corrected this in the text.

8. There are some presentation problems in Fig. 2. In Fig. 2h, the overlays are not transparent enough and become very hard to distinguish where they overlap. For example, does the "red" group have a tail of high-perforin-expressing cells, or not? Increasing transparency or changing the order of the overlays could help. Also, the results in Fig. 2j would be more compelling at a larger size.

We are grateful for the helpful feedback. We have attempted to improve the quality of the graphs to allow better discrimination between the overlays. We have also increased the size of the images in 2j.

9. At the bottom of p. 8, the tSNE plot (Fig. 3f) shows that subsets within the ETP population are not discretely separated, but it does not show that the ETP population is "homogeneous".

Agreed, we have changed the text accordingly.

10. At the bottom of p. 10, a more involved line of logic appears to have been compressed to the point where it is obscure. It is explained in the Discussion, but if the connection is mentioned at this point in the Results, it would be helpful to spell out that these cells and the gamma delta cells both express Ccr9, which has been shown to important for siLP homing

We have added text to clarify the potential role of CCR9.

11. On p. 17, lines 9-10, this should be one sentence rather than two.

Now corrected.

Reviewer #2

(Remarks to the Author)
In this manuscript Ferreira and colleagues address multiple important questions in ILC2 biology including the origin of ILC2 and the transcriptional mechanisms that lead to ILC2 lineage determination. There are many different points made in the manuscript, as outlined below, but the most critical of these (in the opinion of this reviewer) is that the transcription factor RORa plays a role in controlling the ILC2 versus T lymphocyte fate choice in the embryonic thymus by directly regulating expression of Nfil3 and Id2 in cells that would otherwise extinguish their expression due to high expression of Bcl11b. The authors do provide some evidence that is consistent with this model including binding of RORa to the Nfil3 and Id2 genes in ILC2 and increased T cell differentiation in vitro in cultures seeded with RORa-deficient ETPs after culture in IL-2 + IL-33. However, they fail to prove the point by showing that single progenitor cells with the option of T lymphocyte or ILC2 potential, chose the T cell fate in the absence of RORa. They need to test the frequency of ILC2 and T cell progenitors at the single cell level to rule out outgrowth of T cells in the absence of ILC2.

There is a lot of interesting data presented in this manuscript but it is put together in a way that does not flow coherently and none of the points are demonstrated as well as they should be. The authors make the following conclusions: 1) that ILC2 arise from ETP and DN2 cells in the fetal thymus (E15.5 and E19.5) and can be identified using 5X polychromILC reporter mice; 2) that thymic ILC2 expand in IL-33 and that stromal cells are the major embryonic thymic cell type making IL-33; 3) that thymic ILC2 preferentially populate the intestine lamina propria; and 4) that RORa is required to suppress the T cell fate from ETPs and 5) that RORa binds to DNA near

Nfil3 and Id2 as well as at Th2 cytokine loci to promote ILC2 development.

Additional comments:

1) The authors identified and characterized sub populations of T and ILC2 progenitors in the embryonic thymus using two separate methods, however in the main text they do not sufficiently show that these populations are the same. Figure 1 uses RNA-seq to describe the transcriptome of the 4 subpopulations identified by surface marker expression and Figure 2 identifies 4 populations based on BC11b- and Id2-reporter expression. In the main figures they do not correlate the populations from Figure 1 and Figure 2, leaving some doubt about the legitimacy of an ILC2 population, identified via transcriptomics in figure 1, and the BC11b/Id2 double positive population, identified in figure 2, being the same subset of cells. The authors attempt to rectify this in the supplement, by comparing the transcriptomes of the populations identified in both figures. To confidently compare the populations, there should be an analysis showing the relative correlation of the transcriptomes of cell populations from both figures.

We are surprised by this comment since we have used many more markers than most studies to define the ILC2 populations - at the levels of gene expression, cell surface marker staining, and transcription factor reporter expression. We cross-compared the highly expressed genes to discriminate ILC2 in conjunction with TF expression. Obviously, the more genes that are included the more likely there is to be overlap due to the close relationship of all these lymphocytes progenitors, which is one of the reasons that specific low-number populations haven't been identified previously. Furthermore, all the analyses from Figure 2 onwards use the cells as defined by TF and surface marker expression, and these are more critically separated than in many other studies where TF reporter mice were not used.

2) The authors use FTOC to characterize the 4 populations that they identify by Id2- and Bcl11b-reporter expression. They indicate that they were unable to perform single cell experiments under these conditions and therefore only bulk populations are analyzed. To demonstrate a single cell origin for ILC2 and T cells they really should do single cell experiments, and this should be feasible in OP9-DL1 cultures (for example in Figure 6). However, the result is not unexpected given previous studies from the Sun lab, which are not referenced in this manuscript but should be (Qian L., et al., J Exp. Med. 2019 Apr 1:21(4):884-899. And Wang H, et al. J Immunol. 2017 Apr15:198(8):3149-3158.).

We were already aware that ILC2 could arise from single cells isolated from adult thymus, from our own work published in Nat Immunol 2012, and we cited this manuscript. We will now also include the Wang ms from 2017 that confirms and extends our original findings. We wanted to build on these initial descriptions by using the more physiological in vivo FTOC model to investigate thymic ILC progenitor development. We have done this successfully, but this model is not capable of supporting single clone transfer. Given the existing data from ourselves (Wong et al 2012) and Wang et al., we do not believe it is necessary to recapitulate these data, and have now included the citation of Wang et al and Qian et al.

3) The points about thymic stromal IL-33 being sufficient to promote ILC2 development seem out of place in this manuscript. The authors show, using
IL-33 reporter mice that the major IL-33 producing cells in the embryonic thymus are EpCAM$^-$ thymic stromal cells. However, in the supplemental material, they show that IL-33 is not required for thymic ILC2 development. Thus, this data does not provide anything important for the paper or impactful for the development of ILC2 in the embryo.

We do not say that IL-33 is sufficient to promote ILC2 development in the embryonic thymus. It is known that IL-33 promotes ILC2 expansion; however, ILC2s are present in IL-33 deficient mice (Hardman et al., 2013). Since IL-33-producing stromal cells can stimulate local ILC2 proliferation in adipose tissue (Rana MJB et al., 2019), we aimed to investigate if the same could be happening in the thymus. We believe these results will be of interest to the field and stimulate further investigations into the still poorly understood microenvironmental factors that regulate ILC differentiation.

We have also added new data that show a closer proximity of ILC2 to IL-33-expressing cells, than pro-T cells to IL-33-expressing cells. See Fig. 4h and i.

4) The data showing that thymic ILC2 preferentially colonize the intestinal lamina propria is also not fully developed and does not contribute substantially the major points raised in this manuscript. The authors demonstrate this intestinal LP homing preference by transplanting fetal thymus under the kidney capsule of adult mice and therefore it is not clear whether their natural tropism during fetal life is being revealed. Moreover, the experiment of co-transplantation of fetal thymic and bone marrow derived ILCs does not show a major tissue tropism for thymic-derived ILC2 (they are present at about 20% of control in both the iLP and lung). These experiments have very different contexts and it is difficult to interpret how these tropisms might reflect normal

migration patterns. In this respect, again the authors should have referenced Sun et al, who show that ILC2 fate-mapped using LckCre are present in bone marrow, lung, and blood but not detectably in the siLP suggesting that thymic derived ILC2 can seed these tissues. The point of the authors here might have been to show Ccr9 on ILC2 derived from the thymus.

We believe that the transplantation of fetal thymus under the kidney capsules is the best approach we could use to access the capacity of thymic ILC2p to emigrate from the thymus and repopulate peripheral tissues. This approach clearly demonstrated that ILC2p developing in the embryonic thymus have the capacity to produce ILC2 and that these in situ-developing cells migrate preferentially from the thymus to the lamina propria.

In the other experiment (shown in Fig 5h-j) we wished to directly compare the ability of BM ILC2p with the ability of thymic ILC2p to repopulate peripheral tissues. In this case the thymic ILC2p have been harvested and the injected into the recipient mice (alongside BM ILC2p), and have not emerged in the same way as ILC2p from the more physiologically relevant kidney capsule model. As such we believe it would be erroneous to compare these models with each other. However, the BM and thymus-derived single cells that were used to reconstitute the mice have been obtained in a comparable way. Consequently, these two distinct approaches answer the questions they were designed to address, but they are not directly comparable due to their differing sources of the cells.

Minor comments:

1) The ATAC-seq and ChIP-seq data are presented as overlaid data that is difficult to follow for individual parameters. It would be better to present these as individual tracks stacked on top of each other. (For example see Shih et al., Cell 165(5):1120-1133, 2016).

In response to the reviewer we have added the stacked data to the supplementary data as new Figures S4, S5 and S6.

2) The ILC versus T cell fate decision has also been shown to involve the Id2-E protein axis as mentioned in this paper but there were no references given. Miyazaki M et al, Immunity 2017, 46(5):818-834 should have been mentioned and perhaps Xu W et al, Blood 2013, 121(9):1534-1542, which doesn't identify ILC2 but shows a good single cell analysis for T cell/ILC fate.

As requested by the reviewer we have added these papers to our manuscript.

Reviewer #3

(Remarks to the Author)
Ferreira and colleagues study the role for RORa in the generation of T cells versus ILC2 from early T cell precursors (ETP) in the fetal thymus. ETP are heterogeneous and can give rise to T cells as well as NK cells and DC in vitro. By using recently developed transcription factor (TF) reporter mice (polychromILC) that can read out expression of key TFs (Bcl11b, Id2, RORa, Gata3, Rorg), the authors dissect DN1 cells in the fetal thymus and and show that this population contains ETP but also NK and ILC2 precursors based on differential expression of Bcl11b and Id2. They further show that Bcl11b-Id2- cells can give rise to Bcl11b+Id2+ ILC2P as well as Bcl11b-Id2+ NKp in fetal thymic organ culture. As IL-33 can expand mature ILC2, the authors study IL-33 expressing cells using an IL-33 reporter mouse strain and document IL-33+ stromal cells in the fetal thymus; they further show that adding IL-33 to FTOC increases expression of Id2, Gata3 and CD25 on ILC2P populations. The authors
next graft fetal thymus from polychromILC mice to Rag2-/-/gc-/- recipients and detect progeny in several organs which include ILC2 primarily in the gut. They compare the capacity of ILC2P from bone marrow versus thymus to repopulate Rag2-/-/gc-/- recipients and show that both are competent although bone marrow cells are more efficient. Since the authors find that RORa is differentially between Bcl11b-Id2- ETP and Bcl11b+Id2+ ILC2P, they next attempt to dissect the role for RORa in ILC2 versus T cell development in the thymus. Using OP9 stromal based cultures the authors find that RORa-deficient ETP can generate T cells but not ILC2 (as expected) while addition of IL-33 to these cultures promotes ILC2 at the expense of T cells in control ETP but not RORa-deficient ETP. The authors conclude that RORa not only promotes ILC2 development but reciprocally represses T cell development. The authors next take advantage of T2A sequences in their RORa and Gata3 reporter constructs to study
DNA binding sites of these TFs in mature ILC2. They use this dataset to compare with ATACseq accessibility profiles in fetal thymus ETP, ILC2P, NKp and DN2/3 cells to construct a TF interaction map between RORa, Gata3, Bcl11b, Id2 and Nfil3. Essentially, the model proposes that RORa competes with Bcl11b to regulate T cell

versus ILC2 development. In the case of T cells, the absence of RORa allows Bcl11b to repress Id2/Nfil3-mediated ILC development, while in ILC2, RORa competes with Bcl11b thereby allowing Id2/Nfil3 expression at the expense of T cells.

This is an elegant work to dissect the complex TF networks that operate during early T cell and ILC development.

1) The authors carefully assess the molecular signatures of fetal thymus DN1 cells at different embryonic stages. The scRNAseq and analysis of polychromILC mice suggest four or five major subsets (depending on age) including ETP, ILC2P, NKp and gd T cell-like cells. These can be visualized in polychromILC reporter mice by their differential expression of Bcl11b and Id2. One subset (Bcl11b+Id2-) is not discussed although the authors have performed RNA sequencing on this population. These cells at E15.5 and E19.5 appear to have high Gata3 levels (Figure2) but lack RORa suggesting that they are T cells. A fifth population that are proposed as gd T cells (Bcl11loId2lo) are related to this Bcl11b+Id2- subset?

We haven't focused on these populations because we aimed to study ILC development, but we have added text to clarify this point. Our RNA-seq data indicate that both the Bcl11b+Id2- and Bcl11loId2lo cell populations correlate to gd T cells. Indeed, previous reports have described similar gd T cell precursor in the DN1 subset of newborn mice (Spidale et al., 2018) and we now cite this paper.

2) The authors describe the ILC2P and NKp as precursors although their molecular signature clearly shows evidence of a mature phenotype (cytokine expression and relevant cytokine receptors to activate these cells in tissues). Most of the ILC2P appear to co-express mature cell markers (Supp fig 4e). This opens up the possibility that a fraction of the cells identified in the fetal thymus are relatively mature, even at E15.5. While the authors provide some evidence that these ILC2P and NKp are derived in situ via ETP (FTOC Fig 3), it remains possible that some of these cells gain access to the thymus after developing at other sites. Fetal ILC2 waves have been reported that seed may tissues during the embryonic period. In addition, an immature Bcl11b+Id2+ subset in the bone marrow that lacks mature ILC2 markers (ie: negative for IL-5, IL-13, IL-33R…) has been described. Do cells with this phenotype exist in the fetal thymus? If the almost fully mature ILC2 in the fetal thymus are derived from ETP, one might expect to find a trajectory of cells based on the scRNA seq analysis.

We would politely disagree with the reviewer. Most significantly, we have isolated highly purified ETP based not only on the consensus cell surface markers used with in the field, but also transcription factor reporters that are associated with lymphocyte development and used these to repopulate embryonic thymuses and obtained ILC2p from these cultures. We believe that it is highly unlikely, if not impossible, for mature ILC2 to be contaminating these cultures.

Furthermore, we do observe maturation of the ILC2p from cells that show fewer ILC2 markers. For example, upregulation of ST2, ICOS and IL-7Ra cell surface markers (Fig. 2e and Fig. S2a) and TFs including Gata3, Bcl11b, Id2, Rora (Fig. 1b) – between days E15.5 and E19.5. Furthermore, as highlighted by Rev 1, only a small proportion of thymic ILC2p have IL-13 expression, indicating that they are still maturing.

3) The results in Figure 3 provide strong evidence that ETP can generate ILC2 and NK-like cells in FTOC. Still, the efficiency of this process is not known and if would be helpful if the authors provide absolute numbers of T cells versus ILC2/NK cells that are generated. Also, was IL-33 added to these cultures? While it is unlikely that contamination by Bcl11b+Id2+ cells during the sorting of Bcl11b-Id2- cells can explain the results (especially after transfer of 20 Bcl11b-Id2- cells), it would be interesting to know the expansion capacity of sorted Bcl11b+Id2+ cells and Bcl11b-Id2+ cells in FTOC (Fig 3g, h). How many Bcl11b+Id2+ and Bcl11b-Id2+ cells were transferred in the FTOC and how many cells were recovered?

This is a good point and we thank the reviewer for raising this. Firstly, we did not add IL-33 to the cultures, and this was done to avoid the bias that the reviewer mentions. We have now added text to clarify that IL-33 was not included.

Secondly, as requested by the reviewer we have now include cell numbers in new Figure 3j.

4) The authors study IL-33 expression in the thymus using reporter mice. While about 10-20,000 IL-33+ stromal cells are detected, the analysis of IL-33 KO thymus shows that the 1-2,000 mature ILC2 expressing ST2 are not decreased. It is not clear whether these IL-33+ stroma localize near ILC2 cells in the thymus. Also the authors should comment on why there are apparently 10-fold more cytokine producing cells compared to cytokine responsive cells which contrasts with other cytokine systems in the thymus (IL-7 for example).

15

The biology of IL-33 is complex (Moussion et al., 2008; Scott et al., 2018)., and unlike IL-7 it is not

secreted, but is released by still obscure mechanisms that include cell stress, damage or death. Also, in contrast to IL-7, IL-33 is often observed at low levels in epithelial cells and is stored to act as an alarmin. Consequently, it is difficult to compare IL-33 with IL-7 pathways.

We have now generated new data regarding the colocalization of ILC2 and IL-33+ stromal cells. We analysed dual Bcl11b-tdTom x Id2-BFP mice with anti-IL-33 in fetal thymus by confocal microscopy. This analysis identified a closer proximity of ILC2 to IL-33-expressing cells, than pro-T cells to IL-33-expressing cells. See Fig. 4h and i. These data also demonstrated that a large proportion of IL-33^{+} cells were actually localised around the edge of the thymus (see top of Fig 4h).

5) The authors propose a model for TF control of T versus ILC2 development with a critical role for RORa. This is in part based on Figure 6 which analyzes OP9 cultures of WT and RORa-deficient ETP. The authors conclude that RORa not only promotes ILC2 development but reciprocally represses T cell development. There are several points that still need to be addressed. First, it is not clear what cells are being studied in this experiment.

(1)The authors use 'ETP' but it is not clear exactly what markers have been used to isolate ETP from WT and RORA-deficient thymus and whether similarly isolated ETP from polychromILC represent a pure population of Bcl11b-Id2- cells (or not). (2)This is important because if ETP from WT mice contain even a few ILC2P then these cells can explain the result. Second, assuming that the ETP are not containing any ILC2P, then an alternative explanation for the results must be excluded. It remains possible that ILC2 expansion in the presence ofIL-33 in WT cultures have a suppressive effect on T cell development from ETP. The large expansion of IL-33 stimulated ILC2 would block T cell development and this would not occur in RORa-deficient cultures since ILC2 do not expand (perhaps due to lack of IL-33R?). (3)Finally, there is no direct evidence that RORa represses T cell fate. RORa-deficient ETP generate T cells to a similar extent as WT ETP : no evidence for de-repression by RORa. The authors would need to over-express RORa in ETP and show that this represses T cell development.

(1) WT ETPs are defined as DN1 c-Kit-high cells, and as discussed above, Bcl11b-negative Id2-negative ETPs are DN1 c-Kit-high cells. We have added these gating strategies as new supplementary data Figure S3a.

(2) Please see response above. We believe it is highly improbable that ILC2p contamination would explain the results. We would further emphasis that in other experiments where we have purified ETP from 5x polychromILC mice and used the array of TF reporters to allow us to select for ETP we have still observed the development of ILC2. We believe these data are consistent and argue for ILC2 arising from ETP not from contaminants

(3) This is a very good point and we have now performed experiments in which we have over-expressed RORa in ETP and assessed their ability to develop into T cells or ILC2. The resulting data are highly supportive of our hypothesis and we have included them in the main figure 6 and supplementary figure 3. Transduction of ETPs with a retrovirus expressing RORa resulted in a reduction in the capacity of ETPs to differentiate along the T cell lineage and a shift to ILC2 commitment, even under T cell development conditions (New Fig. S3b and main Figure 6d). Furthermore, in separate experiments using 5x polychromILC mice we confirmed that the ICOS+CD44+ cells were Gata3^{hi}Rora^{+}Bcl11b^{+}Id2^{+} ILC2s, and the ICOS^{-}CD44^{-} cells were Gata3^{low}Rora^{-}Bcl11b^{-}Id2^{-} pro-T cells (new Fig. S3c).

6) While the authors model is interesting, one major question remains: how does RORa compete out Bcl11b to allow ILC2 development? The site in Id2 where Bcl11b may repress expression (+40 Kb downstream) is not bound by RORa.

We would politely disagree with the reviewer. The model as proposed does not require competition between Rora and Bcl11b.

Within the circuit, NK cells develop when Bcl11b is not expressed. However, Bcl11b is expressed by both T cells and ILC2. In this context, the presence of RORa in ILC2, but not in T cells, supports the co-expression of Id2 and Nfil3 which repress T cell fate and favour ILC2 commitment (Fig. 7e). Thus, in the absence of Notch signalling, Bcl11b is not expressed, and consequently Id2/Nfil3 dominate to drive NK cell production. However, when Notch signalling is present, Bcl11b is switched on (with other TFs) and dampens Nfil3/Id2 expression, resulting in reinforcement of the T cell pathway by E proteins. By contrast, if RORa is also switched on it serves to override the Nfil3/Id2 repression, thereby creating balanced expression of Bcl11b and Id2. The balanced state permits ILC2 differentiation while simultaneously preventing the E proteins from inducing T cell development.

Minor comments

1) the confocal analysis of ILC2 'foci' requires quantification.

We have now quantified the localisation of ILC2.

Decision Letter, first revision:

Subject: Nature Immunology - NI-A29357A pre-edit

Message: Our ref: NI-A29357A

12th Oct 2020

Dear Andrew & Ana,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Immunology manuscript, "RORα expression is a critical checkpoint for the bifurcation of the T cell and ILC2 lineages in the embryonic thymus" (NI-A29357A).

I am attaching the edited manuscript. I have made changes marked in tracked-changes, queries in red and comments are embedded throughout the manuscript, so please have the view comments option enabled. My biggest concerns are the length of the piece (>2000 over the typical word limit for articles) and use of protein vs gene nomenclature when presenting your results.

Given that the piece is long, I have only lightly edited the text. To reduce length, I typically ask authors to look for is internal redundancies and to eliminate these. That is, try to refrain from the formula "tell them what you will tell them in the Introduction, tell them in the Results, and tell them what you just told them in the Discussion". The introduction should set the context for the question addressed in the study and why readers should care – ie articulating the significance of the question. Although many papers also include a short synopsis of the major conclusions at the end of the introduction, this is not mandatory. Likewise, I find that many authors include a lot of ongoing discussion of the results within the Results section. These too can be minimized to save space. Third, many authors like to recapitulate the results in the discussion; instead place the new findings in context of the literature and explain how the new results thus plug the previous gap in knowledge and how these findings alter our thinking about the immunological phenomenon that is the focus of the study.

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All references must be cited in numerical order. Place Methods-only references after the Methods section and continue the numbering of the main reference list (i.e., do not start at 1).

Genes must be clearly distinguished from gene products (e.g., "gene Abc encodes a kinase," not "gene Abc is a kinase"). For genes, provide database-approved official symbols (e.g., NCBI Gene, http://www.ncbi.nlm.nih.gov/gene) for the relevant species the first time each is mentioned; gene aliases may be used thereafter. Italicize gene symbols and functionally defined locus symbols; do not use italics for proteins, noncoding gene products and spelled-out gene names.

Figures and Tables:

All figures and tables, including Extended Data, must be cited in the text in numerical order.

Figure legends should be concise. Begin with a brief title and then describe what is presented in the figure and detail all relevant statistical information, avoiding inappropriate methodological detail.

Shadings or symbols in graphs must be defined in some fashion. We prefer that you use a key within the image; do not include colored symbols in the legend.

All relevant figures must have scale bars (rather than numerical descriptions of magnification).

All relevant figures must have defined error bars.

Red/green color contrasts can confuse our colorblind readers; please consider recoloring figure 2h, if possible.

Graph axes should start at zero and not be altered in scale to exaggerate effects. A 'broken' graph can be used if absolutely necessary due to sizing constraints, but the break must be visually evident and should not impinge on any data points.

All bar graphs should be converted to a dot-plot format or to a box-and-whisker format to show data distribution. All box-plot elements (center line, limits, whiskers, points) should be defined.

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Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Statistics and Reproducibility:

The Methods must include a statistics section where you describe the statistical tests used. For all statistics (including error bars), provide the EXACT n values used to calculate the statistics (reporting individual values rather than a range if n varied among experiments) AND define type of replicates (e.g., cell cultures, technical replicates). Please avoid use of the ambiguous term "biological replicates"; instead state what constituted the replicates (e.g., cell cultures, independent experiments, etc.). For all representative results, indicate number of times experiments were repeated, number of images collected, etc. Indicate statistical tests used, whether the test was one- or two-tailed, exact values for both significant and non-significant P values where relevant, F values and degrees of freedom for all ANOVAs and t-values and degrees of freedom for t-tests.

Reporting Guidelines– Attached you will find an annotated version of the Reporting Summary you submitted, along with a Word document indicating revisions that need to be made in compliance with our reproducibility requirements. These documents detail any changes that will need to be made to the text, and particularly the main and supplementary figure legends, including (but not limited to) details regarding sample

sizes, replication, scale and error bars, and statistics. Please use these documents as a guide when preparing your revision and submit an updated Reporting Summary with your revised manuscript. The Reporting Summary will be published as supplementary material when your manuscript is published.

Please provide an updated version of the Reporting Summary and Editorial Policy Checklist with your final files and include the following statement in the Methods section to indicate where this information can be found: "Further information on research design is available in the Nature Research Reporting Summary linked to this article."

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Supplementary Information:
All Supplementary Information must be submitted in accordance with the instructions in the attached Inventory of Supporting Information, and should fit into one of three categories:

25 EXTENDED DATA: Extended Data are an integral part of the paper and only data that directly contribute to the main message should be presented. These figures will be integrated into the full-text HTML version of your paper and will be appended to the online PDF. There is a limit of 10 Extended Data figures, and each must be referred to in the main text. Each Extended Data figure should be of the same quality as the main figures, and should be supplied at a size that will allow both the figure and legend to be presented on a single legal-sized page. Each figure should be submitted as an individual .jpg, .tif or .eps file with a maximum size of 10 MB each. All Extended Data figure legends must be provided in the attached Inventory of Accessory Information, not in the figure files themselves.

26 SUPPLEMENTARY INFORMATION: Supplementary Information is material that is essential background to the study but which is not practical to include in the printed version of the paper (for example, video files, large data sets and calculations). Each item must be referred to in the main manuscript and detailed in the attached Inventory of Accessory Information. Tables containing large data sets should be in Excel format, with the table number and title included within the body of the table. All textual information and any additional Supplementary Figures (which should be presented with the legends directly below each figure) should be provided as a single, combined PDF. Please note that we cannot accept resupplies of Supplementary Information after the paper has been formally accepted unless there has been a critical scientific error.

All Extended Data must be called you in your manuscript and cited as Extended Data 1, Extended Data 2, etc. Additional Supplementary Figures (if permitted) and other items are not required to be called out in your manuscript text, but should be numerically numbered, starting at one, as Supplementary Figure 1, not SI1, etc.

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Other
28 As mentioned in our previous letter, all corresponding authors on a manuscript should have an ORCID – please visit your account in our manuscript system to link your ORCID to your profile, or to create one if necessary. For more information please see our previous letter or visit www.springernature.com/orcid.

29 Nature Research journals encourage authors to share their step-by-step experimental protocols on a protocol sharing platform of their choice. Nature Research's Protocol Exchange is a free-to-use and open resource for protocols; protocols deposited in Protocol Exchange are citable and can be linked from the published article. More details can found at www.nature.com/protocolexchange/about.

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We ask that you aim to return your revised paper within 14 days. If you have any further questions, please feel free to contact me.

Best regards,

Laurie

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Reviewer #1:
Remarks to the Author:

This is an extremely rich and informative paper that contains much of interest to researchers of ILC and T cell development alike, and especially for those interested in the ontogeny of lymphocyte developmental programs. The authors have very satisfyingly responded to all my previous concerns, and in addition the Rora gain of function experiment (Fig. 6d) is a significant additional piece of support for the authors' model.

I find only a few things remaining to fix in this submission.

1. While the "response to reviewers" statement refers to three separate replicates of genomic ChIP-seq data, and the authors should be congratulated for generating these, there is still nothing about the replicates in the Methods. The figure legends only mention that "the tracks shown are representative of two independent experiments". But there is nothing about assessing reproducibility in the methods for the ChIP-seq or ATAC-seq

results, that might be comparable to the authors' use of ComBat to correct for batch effects in RNA analysis. Thus, the Methods section of the paper still needs some account of how similar the separate replicates were, and whether results from replicates were averaged or added to generate the data shown. The statistical section at the very end could include this account of the handling of genomic results, unless there is a separate section devoted to genomic data processing.

2. A typo: in a new sentence on p. 11, the word "of" was missing, and "such us lungs" should be "such as lungs". The sentence should read, "…suggested the presence OF ILC2, of potential thymic origin, in the peripheral tissues, such AS lungs, but not in the siLP"

Reviewer #2:
Remarks to the Author:
The manuscript by Ferriera et al has been substantially improved by the addition of new data and rewording. There are still four main findings in the paper. The first, using scRNA-seq and 5X polychrome reporter mice to identify populations of ILC2 and NK cells in the fetal thymus before the development of DP thymocytes. We had some initial concerns about this data that seem less substantial in the revised version and are definitely improved by the inclusion of replicated data. The second finding is that these fetal thymus-derived ILC2 preferentially home to the intestine. This observation is supported by the thymic transplant data but less so by the competitive transplant data, and it seems that there could be many interpretations about whether these cells really leave the thymus, home to the intesdting, or elsewhere. The third conclusion is that ILC2 and NK cells localize close to ILC-33 producing cells in the thymus but that IL-33 is not absolutely essential for their development. The fourth conclusion is that RORa promotes fetal thymic ILC2 development by promoting Nfil3 and Id2 expression in the presence of Bcl11b. This conclusion is supported by the observed requirement for RORa in vitro to suppress T cell development in ILC2 differentiation conditions, ectopic RORa to promote ILC2 in T cell conditions and the binding of RORa at the Id2 and Nfil3 genes in lymph node ILC2.

The first and fourth of these conclusions are the most convincing and relevant for understanding ILC2 development. The role of RORa in driving ILC2 differentiation in the fetal thymus likely does involve a role in regulation of Nfil3 and Id2 although here the only evidence directly supporting this is RORa binding at the Nfil3 and Id2 genes in more mature ILC2. The authors should state clearly that they have yet to show a dependency of Id2 on RORa. The authors show expression of Id2 in RORa transduced cells in the Figure S3 but the time point is not indicated and it seems to be long after ILC2 have emerged. Do they have data showing induction of Id2 (reporter) at early time points after ectopic expression of RORa (i.e. when GFP is first detected…maybe 24 to 36 hours but before ILC2 emerge)? This would strongly support an induction of Id2 by RORa.

I had requested that the data supporting this to be presented as it is now shown in Figure S6. I think that this layout is more useful than the overlaid data as shown in Figure 7. I suggest putting Figure S6 in the main part of the paper. Also the data in 6e could be supplemental.

Reviewer #3:

Remarks to the Author:
The authors have submitted a revised manuscript that addresses many of the reviewer comments. In particular, the new experiments over-expressing Rora nicely support the model in which this transcription factor can promote ILC2 cell fate in ETP even under T cell conditions.

I had commented that the ILC2 and NK cell precursors identified by the authors in the fetal thymus had several attributes of mature cells, raising the question as to why these cells are designated as 'precursors'. For example, on page 7 the authors describe embryonic thymus ILC2P as Gata3hiICOS+ST2+IL13+ cells. This certainly seems 'mature'…. The authors themselves do not use a single terminology sometimes referring to these cells as thymic ILC2p (in many places) but then call them ILC2 (when analyzing confocal microscopy). A similar point can be made for NK 'precursors' : referred to as NKP but then called NK cells when analyzing confocal localization. It might be helpful to the reader if there was a consistent terminology. My interpretation of the data presented is that these cells are thymic NK and thymic ILC2 rather than precursors.

Minor comment:
Perhaps I missed it, but the input number of cells (ETP, Id2+Bcl11b+, Id2+Bcl11b-) for Figure 3j is not indicated.

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A29357B

Message: In reply please quote: NI-A29357B

Dear Andrew,

I am delighted to accept your manuscript entitled "RORα is a critical checkpoint for T cell and ILC2 commitment in the embryonic thymus" 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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Sincerely,

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