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Thymic crosstalk restrains the pool of cortical thymic epithelial cells with progenitor properties

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision - 17-Jan-2017

Dear Dr. Alves,

Manuscript ID eji.201746922 entitled "Lympho-epithelial interactions restrain the pool of cortical thymic epithelial cells with progenitor properties.", which you submitted to the European Journal of Immunology, has been reviewed. The comments of the referees are included at the bottom of this letter.

Although both referees have fated your submission highly and referee 1 has recommended publication, referee 2 has requested some revisions to your manuscript. The Executive Editor invites you to respond to as many comments of referee 2 as possible and revise your manuscript accordingly.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

European Journal of Immunology

Once again, thank you for submitting your manuscript to European Journal of Immunology. We look forward to receiving your revision.

Yours sincerely,
Karen Chu

on behalf of Prof. James Di Santo

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Reviewer: 1

Comments to the Author
In their study, Meireles et al examine progenitor activity in postnatal thymic epithelial cells (TEC). The continued production of TEC after birth is of key importance to sustained T-cell production. Moreover, this area has become a key area of thymic research that has resulted in several high profile, and often conflicting, publications. As such, this study is important as it tackles an exciting area of research that requires better understanding.

The experiments described here identify a population of cells with a cTEC phenotype in the postnatal thymus that have clonogenic properties, give rise to cTEC and mTEC in vivo, and are controlled by the process of ongoing T-cell development. These findings are presented in a clear and logical way, and experiments are well controlled.

A key experiment in this study is the demonstration that clonoTEC give rise to cTEC and mTEC progeny in vivo. The frequency of clonoTEC descendants is relatively low, and the authors suggest that this might be a limitation of experimental systems used. It would be useful if the authors could perhaps expand this discussion, and include some discussion on how TEC progenitors that exist within the 'carrier' thymus might limit the development of clonoTEC. In addition, can the authors comment on how long the progeny of clonoTEC are detectable for after in vivo transplantation of RTOC? This is important as the progeny of SSEA1+ stem cells in another study (Sekai et al Immunity 2014) were shown to be detectable for at least one year.

Finally, the authors state that EpCAM1- cells from postnatal thymus do not possess colony forming potential. This is very important, as the work of Ucar et al places TEC progenitors in this fraction. It may be useful to include this data in the manuscript.

Reviewer: 2

Comments to the Author
This manuscript by Alves and his colleagues describes thymic epithelial cell-derived colony-forming cells, which they termed ClonoTECs. The authors show that the ClonoTECs are mainly derived from cTECs, the ClonoTECs contain precursor potential to give rise to cTECs and mTECs, and the colony-forming potential of ClonoTECs decreases along the ontogeny in thymocyte-dependent manner. Based on these results, the authors attempt to conclude that lympho-epithelial interactions restrain the pool of cTECs with progenitor properties. The issue is basically important and the story is potentially interesting. The authors should consider the following issues to improve the manuscript.

1) Regarding the self-renewal capability by the ClonoTECs, the authors should include the data from single-cell re-plating assay. The bulk re-plating results for 3 passages (shown in Figure 1D) are not compelling to claim the self-renewal capability.

2) Regarding the bipotent precursor potential by the ClonoTECs, it is again important to detect the bipotent precursor potential from the single-cell re-plated ClonoTECs. Otherwise, it is always possible to detect the precursor potentials by the mixture of cTEC-producing ClonoTECs and mTEC-producing ClonoTECs.

3) Figure 3 and S3 data are not very clear to show the bipotent potential of cultured ClonoTECs. Also, what is the definition of cTECs and mTECs in these experiments? Do you define them solely by the markers like Ly51, K8, UEA, and Aire? Which markers do you precisely detect when you talk about the cTEC and mTEC traits in Figure 3B? Do Ly51+ cTECs and K8+ cTECs always overlap? How about the frequencies of UEA+ mTECs and Aire+ mTECs? Further, how do you find the location of GFP+ cTECs and mTECs in cortical or medullary microenvironments?

4) It seems interesting to detect the effect of developing thymocytes in the colony-forming potential of ClonoTECs. However, how can you conclude that the cellular interactions are direct? The conclusion of lympho-epithelial interactions (for example, described in the title) requires more careful considerations.

5) The authors describe that the CCRL1-GFP-low cells are “additional subsets of mTECs”• . You should describe more clearly why these cells represent mTECs and not cTECs.

First Revision – authors’ response - 24-Feb-2017

Reviewer 1

1) *“It would be useful if the authors … include some discussion on how TEC progenitors that exist within the 'carrier' thymus might limit the development of clonoTEC. “*

We have discussed this point on **pages 10-11**.

2) *“on how long the progeny of clonoTEC are detectable for after in vivo transplantation of RTOC?“ This is important as the progeny of SSEA1+ stem cells in another study (Sekai et al Immunity 2014) were shown to be detectable for at least one year.*

The progeny of *Clono*TECs within the ectopic thymus was analyzed 4-weeks post-thymic transplantation. We agree with the reviewer that a long-term analysis on their maintenance and lineage potential is required. This point is part of our long-term project that also aims at analysing the functional contribution of isolated TECs progenitors in thymopoiesis. We included this consideration on **page 11**.

3.) *“the authors state that EpCAM1- cells from postnatal thymus do not possess colony forming potential. This is very important, as the work of Ucar et al places TEC progenitors in this fraction. It may be useful to include this data in the manuscript.”*

We have included these data in new Figure S1D (**page 5**).

Reviewer 2

1.) *Regarding the self-renewal capability by the ClonoTECs, the authors should include the data from single-cell re-plating assay. The bulk re-plating results for 3 passages (shown in Figure 1D) are not compelling to claim the self-renewal capability.*

In the revised version we opted by changing the term "***self-renewal***" to "**sustained/continual clonogenic/colony-forming potential*"***, in the abstract, introduction, results and discussion (**pages 2, 4, 8 and 10**).

We add images of live cell immunofluorescence analysis from the original experiments showing that *Clono*TECs from different passages retain colony-forming capacity (revised **Figure 1D)**. Given that the number of input cells (6000) was normalized at the start of each passage and we did not observe an increase in colony-forming cells, these results suggested that continual clonogenic2 capacity (*in vitro* self-renewal) is restricted to a fraction of *Clono*TECs. We now include new single-cell re-plating assay using *Clono*TECs from passage 1 (P1), which reinforce the notion that colonies in subsequent passage remain clonal and that the capacity to re-establish them is restricted to a fraction of *Clono*TECs (**new figure S1E**). We attempted to perform single-cell re-plating assays using cells from a couple of single colonies (n=4), but these experiments were challenging and did not reveal colony-forming units. While the number of cells (*Clono*TEC) per colony varies between 100-200 (based on microscopic analysis), the number of *Clono*TECs that we recovered from single colonies for subsequent cell sorting was reduced. Thus, it was complex to ascertain whether cell-sorting and single-cell replating assay analysed all cells from a single colony. This technical impediment is also common to the justification of point 2 (please read below).

2.) *Regarding the bipotent precursor potential by the ClonoTECs, it is again important to detect the bipotent precursor potential from the single-cell re-plated ClonoTECs. Otherwise, it is always possible to detect the precursor potentials by the mixture of cTEC-producing ClonoTECs and mTEC-producing ClonoTECs.*

a) We discussed this alternative scenario raised by the reviewer in the discussion of our manuscript (**pages 11 and 12**). Yet, in the results section, we originally referred to *Clono*TEC as possible bipotent cells. We have edited the text accordingly (**pages 7- 8**).

"Overall, these results indicate that cTEC-derived *Clono*TECs contain cells with multilineage potential to generate cTECs and/or mTECs upon integration in native thymic microenvironments". We remove the word "*multilineage"* form this sentence (pages 7-8).

"The self-renewal activity and bipotent potential of cTEC-derived *Clono*TECs indicate that TEC progenitors nestle within the cTEC niche". We edited this sentence to "The observations that a fraction cTEC-derived *Clono*TECs maintain their clonogenic potential *in vitro* and generates cTECs and/or mTECs indicate that the cortical niche harbours TEC progenitors." (page 8)

b) We agree with the reviewer that determination of the lineage potential of single-cell derived *Clono*TECs (from a single colony) would permit to dissect whether *Clono*TECs include bipotent and/or heterogeneous subset of cTEC-producing and mTEC-producing *ClonoTECs*. Technical reasons related to the sensitivity of detection of "spiked" *Clono*TECs in RTOCs has however hitherto prevented us to successfully perform these type of experiments with less than the indicated amount of *Clono*TECs (**pages 10-12**).

As we further discussed "This technical limitation is common to several studies using hybrid RTOC, which are composed of predominant embryonic thymic stromal cells mixed with adult TEC subsets purportedly enriched with TEPs [15-17]." So far, the sole study that has revealed true bipotent progenitor activity at the single cell levels was performed with embryonic-derived TECs (Rossi *et al*. Nature 2006). Refined experimental setups are required to study this question in purported postnatal-derived subsets. Furthermore, a part of lineage tracing assays, it would be equally important to unravel whether postnatal TEC progenitors can functionally contribute to thymopoiesis. We include these notions in the discussion (**page 11**).

Yet, and considering that *Clono*TECs include cTEC-producing and mTEC-producing cells, the observations that *Clono*TECs were originally generated from prototypical cTECs suggests that the cortical thymic epithelium compartment harbours cTEC and/or mTEC precursors.

3.) *a) Figure 3 and S3 data are not very clear to show the bipotent potential of cultured ClonoTECs.*

We infer that the limited engraftment of *Clono*TEC *in vivo* prevents a more prominent manifestation of their lineage potential (please read above reply to point 1 of referee 1 and **page 10**).

b) Also, what is the definition of cTECs and mTECs in these experiments? Do you define them solely by the markers like Ly51, K8, UEA, and Aire?

We thank the reviewer for this indication. The description of this section was succinct in the original version and we provided further information in the revised version (**page 7** of results and **page 23** legend of Figure 3)

We could not combine all cTEC (Ly51 and K8) and mTEC (UEA, MHCIIbright and Aire) markers and anti-GFP Ab (to survey *Clono*TEC) in a single analysis due to the limited number of parameters in the immunofluorescence analysis. As such, we performed the analysis in serial sections of the ectopic thymus with the indicated combinations.

We defined cTEC-expressing *Clono*TECs as GFP+ cells that reside within cTEC areas (Ly51+ or K8+) and co-expressed Ly51+ or K8+. We defined mTEC-expressing *Clono*TECs as GFP+ cells that reside within mTEC areas (UEA+ and MHCIIbright) and bound UEA or co-expressed high levels of MHCIIhi.

c) Do Ly51+ cTECs and K8+ cTECs always overlap?

We were not able to combine both Abs in the same staining. In the original version, we stated “a fraction of *Clono*TEC progeny co-expressed cTEC traits, such as K8 and Ly51” (page7)”. We referred as co-expression to GFP+Ly51+ or GFP+K8+. We thank the reviewer for bringing this point and we edited the sentence accordingly “Strikingly, a fraction of *Clono*TEC progeny expressed cTEC traits, such as K8 or Ly51”.

d) Which markers do you precisely detect when you talk about the cTEC and mTEC traits in Figure 3B? Further, how do you find the location of GFP+ cTECs and mTECs in cortical or medullary microenvironments? How about the frequencies of UEA+ mTECs and Aire+ mTECs?

*Clono*TEC-derived cells (GFP+) found within cTEC areas (K8+ or Ly51+) and expressing these markers were scored as cTECs. *Clono*TEC-derived cells (GFP+) found within mTEC areas (UEA+ and MHCIIhi) and binding UEA or expressing high levels of MHCII were scored as mTECs. *Clono*TEC-derived cells (GFP+) found within cTEC or mTEC areas but lacking respective markers were scored as undifferentiated. We add this information in the results and legend (**pages 7 & 23**). We found rare *Clono*TEC-derived cells (GFP+) expressing Aire within the mTEC compartment (**page 7**). We included this in the text, but because their frequency was low we opted by not quantifying on Figure 3 B.

4) *It seems interesting to detect the effect of developing thymocytes in the colony-forming potential of ClonoTECs. However, how can you conclude that the cellular interactions are direct? The conclusion of lympho-epithelial interactions (for example, described in the title) requires more careful considerations.*

We agree with the reviewer that our experiments cannot formally prove the direct impact of thymocyte-derived signals on TECs with colony-forming potential of TECs. It is possible that developing thymocytes can interact with other TECs or non-TEC thymic stromal cells that will in turn impact on TECs with clonogenic potential. We have included this notion in the results (**page 9**) and discussion (**pages 12-13**) sections of the paper. We edited our title to "*Thymic crosstalk restrains the pool of cortical thymic epithelial cells with progenitor properties*"

5) *The authors describe that the CCRL1-GFP-low cells are "additional subsets of mTECs". You should describe more clearly why these cells represent mTECs and not cTECs.*

We have shown previously that CCRL1int cells contain additional subsets of mTECs

(UEA+CD80- and UEA+CD80+ [26]). We have provided this additional information in the results sections of the paper (**page 6**).

Second Editorial Decision - 02-Mar-2017

Dear Dr. Alves,

It is a pleasure to provisionally accept your manuscript entitled "Thymic crosstalk restrains the pool of cortical thymic epithelial cells with progenitor properties." for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Prof. James Di Santo

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