European Journal of Immunology

Supporting Information

for

DOI 10.1002/eji.201546253

Song Baik, Miho Sekai, Yoko Hamazaki, William E. Jenkinson and Graham Anderson

Relb acts downstream of medullary thymic epithelial stem cells and is essential for the emergence of RANK$^+$ medullary epithelial progenitors

European Journal of Immunology

Supporting Information

for

DOI 10.1002/eji.201546253

Song Baik, Miho Sekai, Yoko Hamazaki, William E. Jenkinson and Graham Anderson

Relb acts downstream of medullary thymic epithelial stem cells and is essential for the emergence of RANK$^+$ medullary epithelial progenitors

European Journal of Immunology

Supporting Information

for

DOI 10.1002/eji.201546253

Song Baik, Miho Sekai, Yoko Hamazaki, William E. Jenkinson and Graham Anderson

Relb acts downstream of medullary thymic epithelial stem cells and is essential for the emergence of RANK$^+$ medullary epithelial progenitors

European Journal of Immunology

Supporting Information

for

DOI 10.1002/eji.201546253

Song Baik, Miho Sekai, Yoko Hamazaki, William E. Jenkinson and Graham Anderson

Relb acts downstream of medullary thymic epithelial stem cells and is essential for the emergence of RANK$^+$ medullary epithelial progenitors

European Journal of Immunology

Relb acts downstream of medullary thymic epithelial stem cells and is essential for the emergence of RANK+ medullary epithelial progenitors

Song Baik, Miho Sekai, Yoko Hamazaki, William E. Jenkinson, Graham Anderson

Correspondence: Dr. Graham Anderson, Institute for Biomedical Research, Medical School, University of Birmingham, B15 2TT, United Kingdom

---

Review Timeline:
Submission date: 14-Dec-2015
First Editorial decision: 23-Dec-2015
First Revision received: 15-Jan-2016
Accepted: 21-Jan-2016

Handling Executive Committee member: Prof. Francesco Annunziato

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 - 23-Dec-2015

Dear Dr. Anderson,

Manuscript ID eji.201546253 entitled "Relb Is Dispensable Medullary Lineage Specification But Controls The Generation Of RANK+ Medullary Thymic Epithelial Cell Progenitors", 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 the referees have recommended publication, some revisions to your manuscript have been requested. Therefore, I invite you to respond to the comments of the referees 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 referee(s) to ensure the relevance and

timeliness of the data.

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

Yours sincerely,
Laura Soto Vazquez

on behalf of
Prof. Bernard Malissen

Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu
****
Reviewer: 1

Comments to the Author

Using an elegant and novel reporter mouse model, Baik and colleagues provide the first report precisely defining the role of RANK-signaling in the development of medullary thymic epithelial cells (mTEC). The findings demonstrate that the development of the previously characterised mTEC stem cell (Sekai et al) is independent of RANK signalling and the expression of the thymus master regulator Foxn1. In addition, the manuscript also demonstrates that Relb (i) is required for the generation of RANK-positive mTEC progenitors but (ii) dispensable for mTEC lineage specification, thus detailing a stage specific need for Relb in mTEC lineage maturation.
This is an elegant and concise study providing novel conclusions that are backed by carefully realised phenotypic analyses.

There are a few points that the authors may wish to comment on:
- Page 5, Line 49: The sentence that "...RANK expression is initiated .... in a distinct subset..." could be rephrased as the term "distinct" does not seem to do justice to a likely scenario where cells with a Cld3,4-high RANK-positive phenotype are derived from a cell that is SSEA-1-negative, RANK-negative but Cld3,4-positive. (see also page 6, Line 12).
- Figure 1A: The authors may wish to comment the nature and developmental history of the RANK-positive, Cld3,4-negative, SSEA-1 negative cell population.

- Page 8, Line 46: This sub clause of this sentence appears to be incomplete.
- Figure 2B: The frequency of spike cells (I-Aq)in the RTOC is small after 4 days in culture. The authors could speculate in the discussion whether this is entirely the consequence of the very limited addition of these cells to the re-aggregates or whether this low frequency also reflects a dynamic process with transgenic precursors competing with more mature, age-matched, unseparated, wild type TEC and display a slower expansion rate.

Reviewer: 2

Comments to the Author

This is a very interesting manuscript, which brings several important and novel insights into the still elusive question of “how thymic medulla is formed?”•
The results provide a very important extension of a key study published recently in Immunity (Sekai et al), describing a population of dedicated Cld3,4hi SSEA1+ self-renewing unipotent progenitor cells capable of giving rise to the medullary thymic epithelial (mTEC) lineage.
Although it is well established that mTEC development and medulla formation is controlled by the RANK-mediated NFkB signaling, the exact sequence of events controlling mTEC lineage specification and their subsequent development is still poorly understood, especially at a molecular level.
To better address these questions, Baik et al utilized RANK-Venus reporter mice and analyzed mTEC ontogeny during embryonic development.
Indeed, their data demonstrate that the early Cld3,4hi SSEA1+ mTEC progenitors precede RANK+ mTEC progenitor population and that their generation does not depend on the expression of Foxn1 “the master regulator of thymic epithelial development”. These results therefore collectively suggest that mTEC lineage specification occurs independently of Foxn1 and NFkB signaling. Instead, NFkB signals seem to be critical for the downstream events giving rise to RANK+ mTEC progenitors.

Although the study is brief, the conclusions are innovative and are well supported by experimental evidence. Thus it is well suited for publication in EJI.

I don't have any major comments or concerns, only a few minor points for consideration:

1) Throughout the paper, the authors use the term SSEA1+ mTEC stem cells, first described and characterized by Sekai et al. I believe that this term is a bit unfortunate and should not be used. Although the Cld3,4hi SSEA1+ cell population was found to have a self-renewing potential and can give rise to the whole mTEC lineage, it is, nevertheless, a uni-potent progenitor. One of the hallmarks of stem cells is their

ability to differentiate into several different lineages. Therefore I suggest to refer to these cells as early or earliest self-renewing unipotent mTEC progenitors or “mTEC stem cells”.

2) There are several typing errors throughout the text; e.g. page 8, line 50 "which the enable triggering", etc.

3) Since the report is brief, the authors could include a graphical illustration of the sequence of events in mTEC specification and subsequent development

First Revision – authors’ response - 15-Jan-2016

Reviewer 1.
1.We have now altered the sentences on page 5 line 49 and page 6 line 12 to take into account the reviewers suggestion.
2. We now also comment on the RANK Venus+Cld3,4-SSEA-1- cells (Fig. 1A) on page 5.
3. We have now reworded the sentence on page 8, line 46.
4. We now speculate on the reasons for the low cellular recovery of cells from RTOC (Figure 2D) on page 6 and 7.

Reviewer 2.
1. The author raises the issue of whether SSEA-1+ TEC analysed here and initially described by Sekai et al in Immunity 2014 are actually ‘stem cells’. He indicates that stem-ness is defined by two properties: a) self-renewal and b) the ability to generate different lineages. Regarding the second definition, other studies are in agreement with Sekai et al that unipotent stem cells exist which self-renew and give rise to only one cell lineage. We cite an example of this work as reference 16, Van Keymeulen Nature 2011). We therefore think it is appropriate to use the term mTEC stem cells in our study. Indeed this nomenclature is now becoming widely accepted in the thymus field eg (Ohigashi et al Cell Reports 2015; Hamazaki EJI 2015).
2. We have now corrected the manuscript for typographical errors.
3. Graphical illustration.

Second Editorial Decision 20-Jan-2016

Dear Dr. Anderson,

It is a pleasure to provisionally accept your manuscript entitled "Relb Acts Downstream Of Medullary Thymic Epithelial Stem Cells And Is Essential For The Emergence Of RANK+ Medullary Epithelial Progenitors" for publication in the European Journal of Immunology.

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,
Laura Soto Vazquez

on behalf of
Prof. Francesco Annunziato

Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu