The transcriptional repressor Bcl6 promotes pre-TCR induced thymocyte differentiation and attenuates Notch1 activation

Anisha Solanki, Diana C. Yanez, Ching-In Lau, Jasmine Rowell, Alessandro Barbarulo, Susan Ross, Hemant Sahni and Tessa Crompton
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MS TITLE: The transcriptional repressor Bcl6 promotes pre-TCR induced differentiation to CD4+CD8+ thymocyte and attenuates Notch1 activation

AUTHORS: Anisha Solanki, Diana C Yanez, Ching-In Lau, Jasmine Rowell, Alessandro Barbarulo, Susan Ross, Hemant Sahni, and Tessa Crompton

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. Referee #2 has indicated to me that you can address his/her comments #1 and #3 in the text without further experimentation. He/she would like you to address comment #2 with additional experiments, if possible. If you do not agree with any of the referees' criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

Solanki et al. analszed fetal and adult thymic development in conditional Lck-Cre Bcl6flx mice. In FTOC, they find a reduced cell number and decreased percentage of DP and DN4 cells, but a rise in DN3 stage in Bcl6coKO thymi. icTCRbeta expression was reduced showing that Bcl6 affects the rate of transition from DN3 to ISP and DP cells. Inefficient maturation to DP cells was also found in CD3 treated BcL6coKO FTOC on RAGKO background. RNA sequencing was performed on untreated and CD3-treated DN3 cells revealing a less mature transcriptome in ISP and DP cells and up-regulated Notch-mediated transcription in DP cells. Focussing on Notch signalling, increased expression of NICD was observed in Bcl6-deficient thymocytes. Adult Bcl6-deficient thymi showed a modest reduction in total cell number and defective DN-DP transition as found for fetal thymi. Significant increased apoptosis and reduced Bcl2/1 mRNA expression was detected in Bcl6coKO DN4 cells of adult mice.
Altogether, the authors provide evidence that Bcl6 influences DN3 to DP transition in fetal and adult thymus and restricts Notch 1 signalling, a novel finding which advances understanding of thymic T cell maturation. However, the loss of Bcl6 has no major impact on SP subset distribution in adult mice and results in only a modest reduction in cell number, although Bcl6 mRNA is strongly upregulated in DP cells (Supp Fig.1), a point which is not sufficiently addressed in the discussion; i.e. is Bcl6 expression in DP cells relevant? The regulation of Notch1 signalling by Bcl-6 is interesting and the augmented expression of ICND in absence of Bcl-6 is impressive. The experiments and data are sound and well presented.

Comments for the author

Issues the authors should address:
1) In some, but not all Figures data legends are given; this should be standardized.
2) Figure 1F, Figure 2B, Figure 6D: show CD4/CD8 expression gated on CD3- cells;
i.e. are the DP, iCD8+ and DN4 cells then negative for CD3/TCR expression? The same on Page 17 Methods qRT-PCR: DN4 (CD4-CD8-CD25-CD44-CD3- ?) cells 3) Page 5 …"typically from 12.8% in control…. typically from 16.6.% in control to 12%", are these values really typical? The average values should be given.
4) Western Blots should show molecular weight markers.
5) The authors should complement their studies by showing Bcl-2 protein expression and cell division in DN3/DN4 cells of both mouse lines. They also have to provide a Western Blot/icFACS staining to proof Bcl6 protein deletion in DN3/DN4/DP cells of Bcl6coKO thymus (as non-deleted cells may expand preferentially).
6) In the discussion the role of the ICND in regulating the AKT pathway and survival should be included.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Solanki et al. describes a novel role for Bcl6 at the pre-T cell receptor (TCR) dependent checkpoint during T cell development, wherein CD4-CD8- double negative (DN) thymocytes differentiate and proliferate to give rise to CD4+CD8+ double positive (DP) cells. A slight delay and lower frequency of DP cells is seen in fetal thymuses, and in adult mice that have been synchronize in their differentiation following hydrocortisone treatment. The use of Rag1 and Bcl6 deficient fetal thymus organ cultures treated with anti-CD3 to trigger TCR beta-selection, directly addresses the role of Bcl6 at the pre-TCR checkpoint, showing a slight delay in DN to DP transition. The authors also show that loss of Bcl6 expression, using a conditional deletion under the control of Lck-cre, leads to a set transcriptional changes that feature increases in Notch target genes. Additionally, the authors show that Bcl6 deficient DN thymocytes show increased levels of Notch intracellular domain (NICD), pointing to upregulated Notch activation within the thymus. Of note, the adult thymus phenotype shown Figure 6 A-C is remarkably unimpressive, but the value of the work done by the authors beautifully illustrates the power of examining genetic deficiencies within the developmental context of ontogeny, and as such we can now appreciate a previously

missed role for Bcl6 in thymocyte differentiation. The authors based this analysis of their previous work showing the transcriptional changes induced by beta-selection, where Bcl6 was shown to by upregulated by pre-TCR signaling. Overall, the present work provides clear evidence that Bcl6 serves to support pre-TCR dependent differentiation outcomes. However, what is missing is a direct connection between the observed increases in Notch activation and functional consequences. Nevertheless, the work is elegantly performed and the findings support their conclusions.

Comments for the author

1- The results shown in Figure 4D are quite striking, revealing increased levels of NICD within fetal thymocytes lacking Bcl6. However, it would be important to show some kind of functional significance to the increase levels of NICD. The authors do show that treatment with gamma-secretase inhibitors (GSI) leads to a reduction in NICD levels of treated FTOCs. To extend these findings and provide a functional read out, one suggestion is to perform a dose response to curve to GSI treatments, and see whether the developmental block typically observed with GSI treatment is blunted in the Bcl6 deficient thymuses. Albeit, this rescue in the differentiation may be affected by the reported requirement for Bcl6 in DN to DP transition, but then again if the block in DN to DP is due to increased Notch levels, then the use of lower amounts of GSI may still reveal a rescue in the Bcl6 samples.
2- Figures 1 and 6 show that DN4 cells have reduced % of TCRb+ cells. It would be interesting to know whether these changes are accompanied by increased in the % of TCRgd+ cells at the DN4 stage, which would support the notion that Bcl6 plays a role in gd vs ab T lineage bifurcation.
3- The authors point out that IL-7 signaling is known to downregulate Bcl6 expression, which helps to further explain the observation that transgenically increased levels of IL-7 lead to a block in thymocyte differentiation. The authors may want to add this notion to their discussion.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Solanki provides details about the role of BCL-6 in the early stages of T cell development. The pre-TCR represent a critical stage in T cell development, primarily signal transduction is involved in the transition of DN to DP cells. However, the precise mechanism involved in this pathway still needs to be elucidated. The authors identify that BCL-6 is up-regulated during pre-TCR signalling and provide experimental evidence that it plays a role in early T cell development which may, in part, involve attenuating Notch 1 activation.

Comments for the author

Only minor comments-
Fig 4 D- is it possible to provide quantification analysis Discussion- it seems in part a reiteration of the results and not providing a biological explanation of their data. What other role does BCL6 play in leukocyte biology?

First revision

Author response to reviewers' comments

As a result of the coronavirus pandemic our animal house, flow cytometry facility and labs are not open, and we are unable to complete the precise additional experiments requested by the reviewers, but we have tried to address all the reviewers' comments using different experimental approaches or by adding data we had already generated but not included in the earlier version of our manuscript. Text changes in the manuscript are highlighted in yellow. Before preparing this revision we wrote to the Editor with suggestions of how we could modify the manuscript given that our lab facilities are still closed, and were told to submit the revision with these changes. The suggestions we made to the Editor prior to making the revisions are

written in italics in red below, whereas our response to the reviewers comments and the detail of changes we made are given in blue in bold. We have added new data which we believe addresses all comments.

Reviewer 1 Advance Summary and Potential Significance to Field:
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Issues the authors should address:

1) In some, but not all Figures data legends are given; this should be standardized.
We have made text changes to standardize the figure legends.

2) Figure 1F, Figure 2B, Figure 6D: show CD4/CD8 expression gated on CD3- cells; i.e. are the DP, iCD8+ and DN4 cells then negative for CD3/TCR expression? The same on Page 17 Methods qRT-PCR: DN4 (CD4-CD8-CD25-CD44-CD3- ?) cells
We gated out CD3+ cells in order to exclude any mature CD8SP cells from our CD8ISP gate and to exclude gammadelta T-cells from the DN gates. ISP are cell surface CD3- , but in adult thymus CD3+CD8SP cells are present, and CD8+gammadelta cells may be present in both fetal and adult thymus. Gammadelta cells would fall into the DN4 gate if they were not excluded by gating out CD3+. iCD8+ and DN4 cells do stain negative for cell surface CD3. We can make text changes to explain the gating.
We now have explained the gating in the methods and clarified the staining in the figure legends. Staining showing cell surface CD3 against intracellular TCRbeta is also now included in the supplementary data (Figure S2 and S3).

3) Page 5 …"typically from 12.8% in control…. typically from 16.6.% in control to 12%", are these values really typical? The average values should be given.
The average values are shown in the chart which accompanies the plots. We will remove the word typical.
We have removed the word typical as the reviewer suggests. Average values are given in the figure.

4) Western Blots should show molecular weight markers.
We will show molecular weight markers.
We have added molecular weight markers to the Western Blots.

5) The authors should complement their studies by showing Bcl-2 protein expression and cell division in DN3/DN4 cells of both mouse lines.
We will show Bcl2 expression in the CD25+DN, ISP and DP populations from Rag1-/-Bcl6coKO and Rag1-/-control. We will show staining for Bcl2 expression in DN3, DN4 and DP from Bcl6coKO and

control.
To address cell division: We will show staining for CyclinB1 (as a measure of cell cycle progression) in DN3/DN4 from E16.5 and adult. This does not show significant differences, consistent with very high cell division in embryo where the thymus is growing exponentially, and with the small difference in thymocyte number in the adult. This data supports the notion that the conditional knockout is affecting differentiation and survival induced by pre-TCR signalling.
We have added *Bcl2* RNA-seq expression data to Figure 3D and results page 9 (highlighted), showing increased expression in the Bcl6coKO. We have added Bcl2 protein expression data (staining) showing increased expression in DN3, DN4, ISP and DP in Bcl6coKO compared to control (Fig. 6D).
We have added staining for intracellular Cyclin B1 expression for DN3, DN4, ISP and DP populations for E16.5 (Fig. S2B) and adult (Fig. S3A). These data show a high proportion of cells stain positive on E16.5, consistent with the rapid growth of the thymus, but no significant differences between Bcl6coKO or control in any population in E16.5 and adult.

They also have to provide
a Western Blot/icFACS staining to proof Bcl6 protein deletion in DN3/DN4/DP cells of Bcl6coKO thymus (as non-deleted cells may expand preferentially).

The Bcl6floxed allele that we used to conditionally delete has loxP sites which delete exons 7- 9 (which encode the zinc finger domain). We have compared exon usage in out RNAseq datasets, and this shows very clearly that exons 7-9 are deleted from CD25+DN, ISP and DP populations. Thus, we have shown that it is not the case that non-deleted cells have expanded to give rise to the DP population **(although the DP population in the coKO does show higher expression than ISP).**
We have included the expression of exons 7-9 from the RNAseq dataset in Figure 2H. This shows deletion of the 3 floxed alleles.

6) In the discussion the role of the ICND in regulating the AKT pathway and survival should be included.
We will add this.
This is now discussed in Discussion.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript by Solanki et al. describes a novel role for Bcl6 at the pre-T cell receptor (TCR) dependent checkpoint during T cell development, wherein CD4-CD8- double negative (DN) thymocytes differentiate and proliferate to give rise to CD4+CD8+ double positive (DP) cells. A slight delay and lower frequency of DP cells is seen in fetal thymuses, and in adult mice that have been synchronize in their differentiation following hydrocortisone treatment. The use of Rag1 and Bcl6 deficient fetal thymus organ cultures treated with anti-CD3 to trigger TCR beta-selection, directly addresses the role of Bcl6 at the pre-TCR checkpoint, showing a slight delay in DN to DP transition. The authors also show that loss of Bcl6 expression, using a conditional deletion under the control of Lck-cre, leads to a set transcriptional changes that feature increases in Notch target genes. Additionally, the authors show that Bcl6 deficient DN thymocytes show increased levels of Notch intracellular domain (NICD), pointing to upregulated Notch activation within the thymus. Of note, the adult thymus phenotype shown Figure 6 A-C is remarkably unimpressive, but the value of the work done by the authors beautifully illustrates the power of examining genetic deficiencies within the developmental context of ontogeny, and as such we can now appreciate a previously missed role for Bcl6 in thymocyte differentiation. The authors based this analysis of their previous work showing the transcriptional changes induced by beta- selection, where Bcl6 was shown to by upregulated by pre-TCR signaling. Overall, the present work provides clear evidence that Bcl6 serves to support pre-TCR dependent differentiation outcomes. However, what is missing is a direct connection between the observed increases in Notch activation and functional consequences. Nevertheless, the work is elegantly performed and the findings support their conclusions.

Reviewer 2 Comments for the Author:

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secretase inhibitors (GSI) leads to a reduction in NICD levels of treated FTOCs.

To extend these findings and provide a functional read out, one suggestion is to perform a dose response to curve to GSI treatments, and see whether the developmental block typically observed with GSI treatment is blunted in the Bcl6 deficient thymuses. Albeit, this rescue in the differentiation may be affected by the reported requirement for Bcl6 in DN to DP transition, but then again, if the block in DN to DP is due to increased Notch levels, then the use of lower amounts of GSI may still reveal a rescue in the Bcl6 samples.
We will include experiments in which we treat Bcl6coKO FTOC with DAPT (GSI) to show recovery in rate of differentiation to DP.
We have added these experiments to Fig. 4G and H. These data indicate that DAPT- treatment increases differentiation to DP in Bcl6coKO FTOC.

2- Figures 1 and 6 show that DN4 cells have reduced % of TCRb+ cells. It would be interesting to know whether these changes are accompanied by increased in the % of TCRgd+ cells at the DN4 stage, which would support the notion that Bcl6 plays a role in gd vs ab T lineage bifurcation.
We have not stained our samples with anti-TCRgammadelta, but we can provide data to address this issue by gating on CD3+icTCRbeta- cells because all CD3+ cells that do not express TCRbeta are TCRgammadelta+. This analysis shows that there is no increase in the proportion of TCRgammadelta+ cells (CD3+TCRbeta-) in E16.5 and in adult. We therefore did not detect an influence of Bcl6 on gammadelta vs alphabeta bifurcation. We have not attempted to further dissect gammadelta T-cell development in the absence of Bcl6 because we believe it is beyond the scope of our study: our manuscript is about alphabeta T-cell development, and this is clear in the introduction.
We have added data for E16.5 (Supplementary Figure 2A) and adult (Supplementary Figure 3B), showing that we did not detect a significant difference in the proportion of CD3+icTCRlll- cells (which represent the lllllllllpopulation) in Bcl6coKO compared to control.

3- The authors point out that IL-7 signaling is known to downregulate Bcl6 expression, which helps to further explain the observation that transgenically increased levels of IL-7 lead to a block in thymocyte differentiation. The authors may want to add this notion to their discussion.
We will add this to the discussion.
This is added to the Discussion.

Reviewer 3 Advance Summary and Potential Significance to Field:
The manuscript by Solanki provides details about the role of BCL-6 in the early stages of T cell development. The pre-TCR represent a critical stage in T cell development, primarily signal transduction is involved in the transition of DN to DP cells. However, the precise mechanism involved in this pathway still needs to be elucidated. The authors identify that BCL-6 is up-regulated during pre-TCR signalling and provide experimental evidence that it plays a role in early T cell development which may, in part, involve attenuating Notch 1 activation.

Reviewer 3 Comments for the Author:
Only minor comments-
Fig 4 D- is it possible to provide quantification analysis
We will use image J for quantification.
The quantification is added to the figure legend and discussed in the text, and explained the quantification in the methods.

Discussion- it seems in part a reiteration of the results and not providing a biological explanation of their data. What other role does BCL6 play in leukocyte biology?
We will modify the discussion as suggested.
We have changed the discussion to reduce reiteration of the results and include a better discussion of the context of our data.

Second decision letter

MS ID#: DEVELOP/2020/192203

MS TITLE: The transcriptional repressor Bcl6 promotes pre-TCR induced differentiation to CD4+CD8+ thymocyte and attenuates Notch1 activation

AUTHORS: Anisha Solanki, Diana C Yanez, Ching-In Lau, Jasmine Rowell, Alessandro Barbarulo, Susan Ross, Hemant Sahni, and Tessa Crompton
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors have addressed the suggested issues to satisfaction and I recommend publication of the revised and improved manuscript.

Comments for the author

I have no further suggestions to the authors.

Reviewer 2

Advance summary and potential significance to field

The authors have fully addressed all the initial concerns and should be commended for their efforts given the difficult times that we are experiencing.

Comments for the author

n/a

Reviewer 3

Advance summary and potential significance to field

The early stages of T cell development represents an important stages of T cell maturation and the authors provide further insight into this pathway of T cell differentiation.

Comments for the author

The authors have addressed my comments.