EMBO
*reports*

# Foxp3 expression in induced regulatory T cells is stabilized by C/EBP in inflammatory environments

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**Review timeline:**

| | |
|---|---|
| Submission date: | 22 February 2018 |
| Editorial Decision: | 9 April 2018 |
| Revision received: | 30 July 2018 |
| Editorial Decision: | 14 September 2018 |
| Revision received: | 20 September 2018 |
| Accepted: | 26 September 2018 |

Editor: Achim Breiling

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 9 April 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires a major revision to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address all in a revised manuscript. As the reports are below, I will not detail them here. We feel, however, that in particular the major and minor points noted by referee #1 (who asks for stronger functional data), and the second point by referee #2 are important, and need to be addressed experimentally.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the

nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature.

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Please also format the references according to EMBO reports style. See:
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Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable. See:
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- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

Please also note that we now mandate that all corresponding authors list an ORCID digital identifier that is linked to their EMBO reports account.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS
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Referee #1:

In this manuscript, Lee at al reported that C/EBP is indispensable for the induction and maintenance of Foxp3 expression through binding to the methyl-CRE sequence in Foxp3 TSDR in TGFb-induced iTreg under inflammatory conditions. Demethylated TSDR in the Foxp3 locus has been demonstrated to be important for Foxp3 maintenance and Treg identity. The study presented in this manuscript showed that methyl-CRE sequence in Foxp3 TSDR bound by C/EBP is critical to ensure Foxp3 induction and maintenance in an inflammatory environment. Although the authors provided a new molecular mechanism by which C/EBP ensures stable Foxp3 expression, the in vitro and in vivo Treg functional assays shown in the current manuscript are either not set up properly or

generated less than compelling results. Without convincing functional data, the current manuscript cannot meet the standard for publication.

Major points:
1, In Figure 2D-G, the author used adoptive T cell transfer colitis model to see whether Foxp3+ populations in C/EBPb-transduced cells are real Treg cells with intact suppressive activity. However, they didn't transfer the same number of Treg because they sorted cells based on GFP+ transduced cells which are cultured under the presence of TGFb and culture supernatant, in which condition the percentage of Foxp3+ cell was not identical (22.3% in MigRI vs 59.2% in C/EBPb transduced T cell).

2, The span of the EAE experiment in Figure 5 should be longer to show the full course of the disease. The differences in clinical scores and Th17 cell percentages are very subtle in the figure.

3, In Figure 6B, it seems that control iTreg had no suppressive function on responder T cells, the authors should do this experiment in the traditional way with different ratios of Tregs to responder T cells to confirm C/EBP transduced iTreg are more suppressive.

4, No in vitro suppression assay was performed with C/EBPb transduced mouse iTregs to show if they are also more suppressive.

Minor points:

1, In Figure 2C, the authors showed Foxp3 expression in FACS-sorted GFP+ and GFP- T cells transduced with C/EBPb or MigRI in the presence of TGFb and culture supernatant, it's highly recommended to show Foxp3 expression in different conditions such as in the presence of anti-IL-4, anti-IFNg or both

2, In Figure 4C-D, the authors should show the methylation status of TSDR since they added 5aza to the culture medium to reduce the methylation CpG across the genome.

3, In Figure 5, the author showed that the stability of iTreg cells is preserved by forced expression of C/EBPb, it will be interesting to see whether the TSDR is still methylated in C/EBPb transduced iTreg cells under this condition.

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Referee #2:

In this manuscript by Lee et al., the authors describe a novel mechanism promoting the stability of induced Foxp3 expression and Treg function. Proinflammatory cytokines IFN-g and IL-4 counteract Foxp3 induction, and retinoic acid (RA) counteracts this suppression of Foxp3 transcription. How RA does this has been an area of debate, as its effects include both the down modulation of IL-4 and IFN-g expression from effector T cells as well as direct cell-intrinsic activity on the Foxp3 transcription. Here, the authors show that RA induces C/EBP expression, and that C/EBP is capable of supporting Foxp3 expression in a cell-intrinsic manner during IL-4 and IFN-g signaling. This is done by overexpressing dominant negative C/EBP in T cells and observing reduced FOXP3 induction (by TGFb and IL-2) in conditions where RA is partially promoting Foxp3 expression. By comparing culture conditions with exogenous IL-4 and IFN-g, neutralizing antibodies against them, and effects on cells retrovirally transduced with the dominant negative C/EBP (GFP+) or not (GFP-), a convincing case is made that C/EBP is acting through a cell intrinsic mechanism. Similarly, when C/EBP was overexpressed by retroviral transduction, Foxp3 induction was enhanced regardless of the presence of IL-4 and IFN-g, and these C/EBP-enforced Treg were superior suppressors in a colitis model. Foxp3 induction in vivo was analyzed in a similar manner with DO11.10/RAG T cell transfer, where induction was enhanced with C/EBP overexpression and reduced with C/EBP antagonist over expression. They provide evidence that C/EBP binds a methylated form of the Foxp3 TSDR enhancer region, which serves as an explanation for enhanced transcription in the presence of a methylated enhancer. Enhanced stability of induced Foxp3 expression in the presence of ectopic C/EBP, in vitro, in an EAE model, and in human T cells.

There are a few points to address to align this work with previous findings.

First, in the introduction and discussion, it is important to make a distinction between iTreg (induced in vitro and not necessarily stable) and pTreg (induced in vivo in the periphery, and more stable due to TSDR demethylation). The authors could add to the discussion that C/EBP could play a role in promoting stable Treg phenotype in vivo, that could ultimately lead to the formation of pTreg with demethylated TSDR. Addressing this experimentally would require a fair amount of new work that would fit better in a new paper. A description of the type of Treg C/EBP facilitates would be "iTreg with increased stability".

Second, for the OVA fed model (Fig 3b), there exist publications describing a role for retinoic acid in the formation of pTreg in this model. In light of this work, wouldn't the C/EBP antagonist reduce the efficacy of pTreg formation? To better understand how the relevant factors are controlling pTreg formation, the authors should repeat these experiments and compare the degree of C/EBP, IL-4, and IFN-g induction in transferred T cells with IV vs oral OVA administration.

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Referee #3:

The manuscript by Lee et al investigates the molecular mechanisms of inducible regulatory T cell (iTreg) induction in the context of inflammatory environments. Here, the authors show that in vitro multiple members of the CCAAT/Enhancer-binding protein (C/EBP) family of transcription factors are induced by retinoic acid (RA), and that overexpression of each of these factors promotes-with varying degrees of efficiency-the expression of Foxp3 in the presence of IFN-γ and IL-4.

Further, using three different models, the authors confirm these results in vivo and show that C/EBP overexpression endows iTregs with greater suppressive capacity. Mechanistically, the manuscript reports C/EBP binding to the methylated Foxp3 TSDR. Finally, the paper provides evidence that this function of C/EBP is conserved in human iTregs.

Although the number and redundancy of the C/EBP family members precludes effective knockout studies, the authors rigorously examined the function of this transcription factor family in iTreg biology both in vitro and in vivo. Addressing the points below would further enhance the quality of the manuscript.

Major Concerns
1) It would be helpful to other investigators to see the data on iTreg induction from Cebpbfl/fl CD4-Cre mice. This would further solidify the point correctly made by the authors in the conclusion about redundancy amongst the C/EBP factors.

Minor Concerns
1) Due to how the qPCR graphs are reported in Figure 1A and Supplementary Figure 1A-C it is difficult to know the relative expression of each of the C/EBP family members in iTregs compared to one another. Commenting on this or showing side-by-side expression data would be helpful. Additionally, any information on the expression of these factors in natural Tregs (nTreg) would also be a useful comparison.

2) iTregs in the intestine can develop into one of several phenotypes with co-expression of Rorγt or GATA3. Co-expression of these transcription factors plays a key role in the suppressive capacity of iTregs, especially at mucosal surfaces. Does C/EBP overexpression alter the expression of these transcription factors in iTregs either in vitro or in vivo?

3) Please state the number of times the colitis experiment in Figure 5D was performed.

4) Scurfy mice, anti-RORγ antibody, and EMSA probes for RORE in the Il17 locus are listed in the methods but don't seem to be used in any of the datasets.

**Reviewer 1**

Major criticism

Comment 1. In Figure 2D-G, the author used adoptive T cell transfer colitis model to see whether Foxp3$^+$ populations in C/EBPβ-transduced cells are real $T_{reg}$ cells with intact suppressive activity. However, they didn't transfer the same number of $T_{reg}$ because they sorted cells based on GFP$^+$ transduced cells which are cultured under the presence of TGFβ and culture supernatant, in which condition the percentage of Foxp3$^+$ cell was not identical (22.3% in MigRI vs 59.2% in C/EBPβ transduced T cell).

Response: As the reviewer pointed out, the experiments associated with Figure 2D-G were initially intended to verify that Foxp3$^+$ populations in C/EBPβ-transduced cells are real $T_{reg}$ cells with intact suppressive activity in the colitis model. Following the reviewer's comments, we tested the suppressive activity of C/EBPβ-transduced $iT_{reg}$ cells sorted based on NGFR$^+$ (virus-transduced) GFP$^+$ (Foxp3-positive). We have now included the data related to IBD colitis model showing weight loss with statistical analysis (Fig 5G), histological scoring (Fig 5H and I) and Foxp3 expression in $iT_{reg}$ cells (Fig 5J) after transferring the same number of $iT_{reg}$ cells. Our results confirm the regulatory activity of C/EBPβ-transduced $iT_{reg}$ cells.

Comment 2a. The span of the EAE experiment in Figure 5 should be longer to show the full course of the disease.
Response: We have conducted pilot study to examine the progress of the disease and found that all mice fell into a moribund state (score: 5) around day 20. That is why we presented data until day 16. However, we agree with the reviewer's comment that tracking the course of the disease until around day 20 is still valid and more informative. We have now added the required data (Fig 5C).

Comment 2b. The differences in clinical scores and Th17 cell percentages are very subtle in the figure.

Response: We thank the reviewer for the comment. Although we have performed additional experiments, the differences in clinical scores and Th17 cell percentages in the EAE model were modest. However, the attenuation of clinical scores and Th17 cell percentages with C/EBPβ-transduced $iT_{reg}$ cells is statistically significant. Moreover, in addition to EAE model, we have now included new data of IBD model, which also supports the enhanced suppressive activity of C/EBPβ-transduced $iT_{reg}$ cells.

Comment 3. In Figure 6B, it seems that control $iT_{reg}$ cells had no suppressive function on responder T cells, the authors should do this experiment in the traditional way with different ratios of $T_{regs}$ to responder T cells to confirm C/EBP transduced $iT_{reg}$ are more suppressive.

Response: We appreciate the reviewer's comment. We performed additional experiments and have replaced the data with a new one. (Fig 6B)

Comment 4. No in vitro suppression assay was performed with C/EBPβ-transduced mouse $iT_{regs}$ to show if they are also more suppressive.

Response: We thank the reviewer for the valuable comment. Following the reviewer's suggestion, we have now included new data demonstrating greater suppressive activity of C/EBPβ-transduced mouse $iT_{reg}$ cells (Fig 5B).

Minor criticism

Comment 1. In Figure 2C, the authors showed Foxp3 expression in FACS-sorted GFP$^+$ and GFP$^-$ T cells transduced with C/EBPβ or MigRI in the presence of TGFβ and culture supernatant, it's highly recommended to show Foxp3 expression in different conditions such as in the presence of anti-IL-4, anti-IFNg or both.

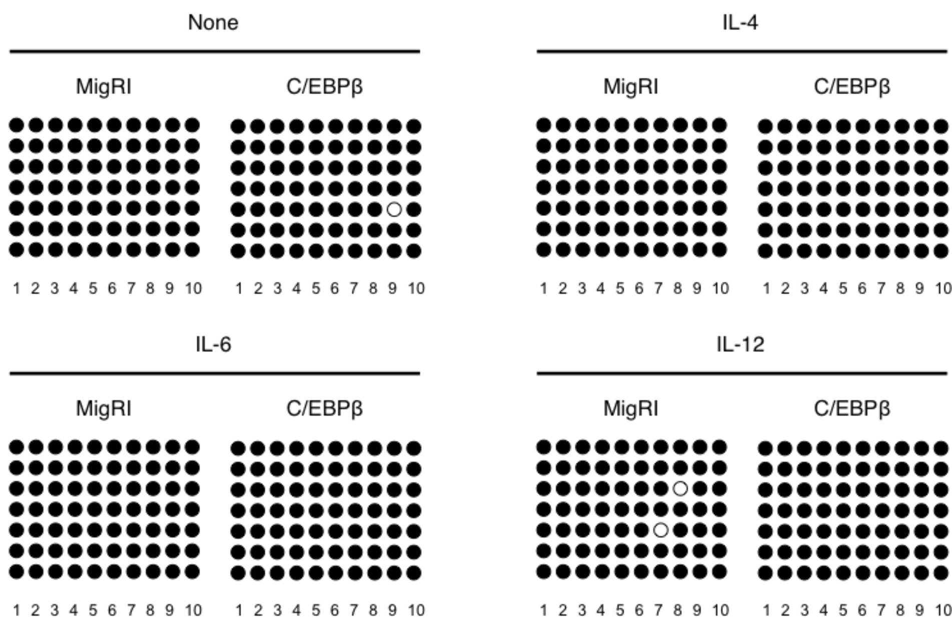
Response: We have now added the result of RT-qPCR showing Foxp3 expression in different conditions (Fig. 2C).

Comment 2. In Figure 4C-D, the authors should show the methylation status of TSDR since they added 5aza to the culture medium to reduce the methylation CpG across the genome.

Response: We have now shown the methylation status of TSDR following addition of 5-Aza (Fig EV 6C)

Comment 3. In Figure 5, the author showed that the stability of $iT_{reg}$ cells is preserved by forced expression of C/EBPβ, it will be interesting to see whether the TSDR is still methylated in C/EBPβ transduced $iT_{reg}$ cells under this condition.

Response: We have now shown the methylation status of TSDR in Fig R1. We did not observe significant demethylation of TSDR in any conditions.
*Fig R1*. Methylation status of TSDR in Fig 5A




**Reviewer 2**

Major criticism

Comment 1a. In the introduction and discussion, it is important to make a distinction between $iT_{reg}$ (induced in vitro and not necessarily stable) and $pT_{reg}$ (induced in vivo in the periphery, and more stable due to TSDR demethylation).

Response: We thank the reviewer for pointing out the imprecise statement. We have edited our manuscript accordingly.

Comment 1b. The authors could add to the discussion that C/EBP could play a role in promoting stable Treg phenotype *in vivo*, that could ultimately lead to the formation of $pT_{reg}$ with demethylated TSDR. Addressing this experimentally would require a fair amount of new work that would fit better in a new paper. A description of the type of $T_{reg}$ C/EBP facilitates would be "$iT_{reg}$ with increased stability".

Response: We thank the reviewer for bringing up this issue. As the reviewer suggested, we have discussed this point in the revised manuscript (page 15, line 8) as follows: As mentioned above, the TSDR of $pT_{reg}$ gradually becomes demethylated during the course of differentiation. The complete

development of $pT_{reg}$ equipped ultimately with demethylated TSDR might need to be preceded by the unique methylation-dependent C/EBP activity to protect Foxp3 expression from inhibitory cytokines at the early stages of its development.

Comment 2. For the OVA fed model (Fig 3b), there exist publications describing a role for retinoic acid in the formation of $pT_{reg}$ in this model. In light of this work, wouldn't the C/EBP antagonist reduce the efficacy of $pT_{reg}$ formation? To better understand how the relevant factors are controlling $pT_{reg}$ formation, the authors should repeat these experiments and compare the degree of C/EBP, IL-4, and IFN-g induction in transferred T cells with IV vs oral OVA administration.

Response: We thank the reviewer for bringing up this point. We addressed this issue by performing additional experiments in which the expression of IFN-γ, IL-4 and C/EBPβ in transferred DO11.10 $CD4^+$ T cells was analyzed by intracellular staining followed by FACS analysis. As expected, we observed that IFN-γ production was greatly repressed to negligible levels in $DO11.10^+CD4^+$ T cells following oral administration compared to i.v injection (Fig R2A). We think that this result supports the role of C/EBP in overcoming the inhibitory effect of inflammatory cytokines during $iT_{reg}$ differentiation. In addition, we found that there is no difference in the C/EBPβ expression level in the transferred $DO11.10^+CD4^+$ T cells between oral administration and i.v injection (Fig R2B).

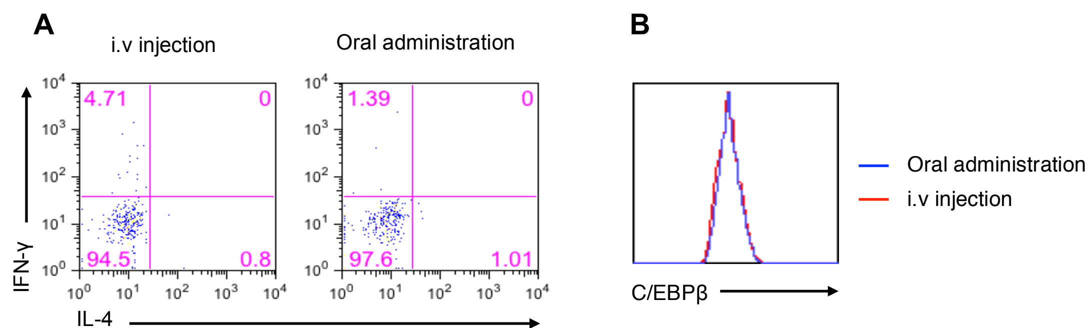



*Fig R2*. (A, B) Flow cytometry of intracellular IFN-γ and IL-4 (A) and C/EBPβ (B) staining of mesenteric lymph node cells of Balb/c mice that received $RAG2^{-/-}$DO11.10 $CD4^+$ naïve T cells. The recipients were immunized via intravenous injection of $OVA_{323-339}$ peptide (20 ug) 1 and 3 d after transfer of transduced cells or fed 1% OVA solution in drinking water for 5 consecutive days. Cells were stimulated with PMA/Ionomycin for 4hr before intracellular staining.

**Reviewer 3**

Major criticism

Comment 1. It would be helpful to other investigators to see the data on $iT_{reg}$ induction from $C/EBP\beta^{fl/fl}$ CD4-Cre mice. This would further solidify the point correctly made by the authors in the conclusion about redundancy amongst the C/EBP factors.
Response: We have now presented the results about $iT_{reg}$ differentiation in $C/EBP\beta^{fl/fl}$ CD4-Cre mice in Fig EV 7.

Minor criticism

Comment 1. Due to how the qPCR graphs are reported in Figure 1A and Supplementary Figure 1A-C it is difficult to know the relative expression of each of the C/EBP family members in $iT_{regs}$ compared to one another. Commenting on this or showing side-by-side expression data would be helpful. Additionally, any information on the expression of these factors in natural $T_{regs}$ ($nT_{reg}$) would also be a useful comparison.

Response: We also agree with the reviewer that it would be beneficial to know the relative expression of each of the C/EBP family members in $iT_{reg}$ cells to estimate their relative contribution to the differentiation and stability of $iT_{reg}$ cells. Following the reviewer's suggestion, we have now presented the expression level of each of the family members as a value normalized to actin (Fig 1A

and Fig EV1). However, given the unequal efficiency of PCR primers of the C/EBP family members, comparing the relative expression levels of family members would be insurmountable in our system and thus, we cannot make a decisive statement about relative expression level.

Comment 2. $iT_{regs}$ in the intestine can develop into one of several phenotypes with co-expression of Rorγt or GATA3. Co-expression of these transcription factors plays a key role in the suppressive capacity of $iT_{regs}$, especially at mucosal surfaces. Does C/EBP overexpression alter the expression of these transcription factors in $iT_{regs}$ either *in vitro* or *in vivo*?

Response: Although we performed additional experiments to study the effect of C/EBPβ on the generation of RORγt$^{+}$ or GATA3$^{+}$ $T_{reg}$ *in vitro*, we did not observe any detectable generation of these cells *in vitro* (Fig R3). To further examine the role of C/EBP for the generation of these cells *in vivo*, we explored $iT_{reg}$ differentiation in lamina propria following oral administration of ovalbumin. However, we were unable to overcome technical hindrances getting enough number of cells in analyzing $iT_{reg}$ differentiation in this model (Fig R4). After the adoptive transfer of DO11.10$^{+}$CD4$^{+}$ T cells, the number of DO11.10$^{+}$CD4$^{+}$ T cells in the small intestine lamina propria was too low to get any conclusive information. We hope that the reviewer appreciates this insurmountable technical difficulty.

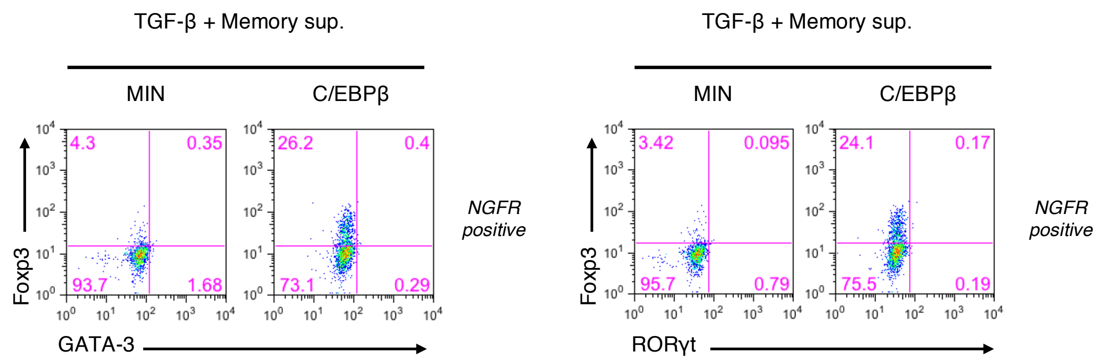



*Fig R3*. Flow cytometry of intracellular GATA-3, RORγt and Foxp3 staining in CD4$^{+}$ naïve T cells transduced with control retrovirus (MIN) or retrovirus encoding C/EBPβ and cultured for 2 d in the presence of memory supernatant and TGF-β. Dot plots are gated for CD4$^{+}$NGFR$^{+}$.

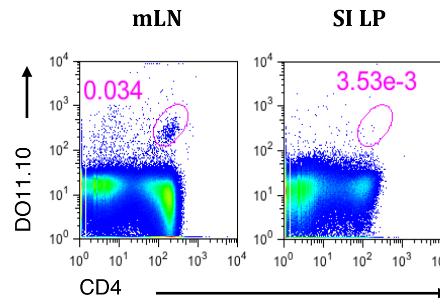



*Fig R4*. Flow cytometry of DO11.10 and CD4 expression in the mLN and small intestine lamina propria (SI LP) of Balb/c mice that received RAG2$^{-/-}$DO11.10 CD4$^{+}$ naïve T cells transduced with control retrovirus (MigRI). The recipients were fed 1% OVA solution in drinking water for 5 consecutive days. There were few DO11.10$^{+}$CD4$^{+}$ T cells in SI LP.

Comment 3. Please state the number of times the colitis experiment in Figure 5D was performed.

Response: We think that you have meant Figure 2D, not Figure 5D on the colitis experiment. We have stated that the colitis experiment was performed XXX times in figure legends as follows: Each time point contains three (circles) or four (squares and triangles) mice in each group

Comment 4. Scurfy mice, anti-RORγ antibody, and EMSA probes for RORE in the Il17 locus are listed in the methods but don't seem to be used in any of the datasets

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Response: Our sincere apology for our careless mistakes. We have deleted all of the irrelevant materials.

2nd Editorial Decision 14 September 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from two of the referees that were asked to re-evaluate your study, you will find below. Referee #3 was not able to re-assess the study, but going through your point-by-point response, I consider his/her concerns as adequately addressed.

As you will see, both referees now support the publication of your manuscript in EMBO reports. Referee #2 has some further suggestions to improve the manuscript, we ask you to address in a final revised version of the manuscript.

Further, I have these editorial requests:

- The abstract is currently slightly too long. Please reduce it to not more than 175 words. Please also provide the abstract written in present tense.

- Regarding data quantification and statistics, can you please check for all diagrams that the number "n" for how many independent experiments (biological replicates) were performed and also the test used to calculate p-values is indicated in the respective figure legends. Please also add a paragraph to the Methods section explaining the statistical analyses used throughout the paper. See: http://embor.embopress.org/authorguide#statisticalanalysis

- Could statistical testing be provided for Fig. EV3B?

- Please provide scale bars for the microscopic images in Figures 2E and 5H. Please indicate the size of the bars in the respective figure legends (not within in the panels - do not write on the scale bars).

- Please add a TOC (table of contents) and page numbers to the Appendix, and name the file 'Appendix'.

- Please format the references according to EMBO reports style. See: http://embor.embopress.org/authorguide#referencesformat

- Please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels).

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

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Referee #1:

In this revised manuscript, Lee and colleagues have adequately addressed all the questions this reviewer raised in the first round. This reviewer is pleased to see the new IBD data, in vitro suppression data, and some minor changes to improve the original manuscript. In the reviewer's opinion, the current version of the manuscript is up to the standard for publication.

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Referee #2:

This is a greatly improved manuscript and my original concerns have been addressed. I can recommend a few minor edits:
1) The y-axis on all plots in each figure should be the same. For example, in Fig 1, the plots should all be 0-100 %. This helps with quickly interpreting the results all together. The p value symbols will still convey the effect sizes. 2) In the abstract, return the text to the original mention of iTreg as this is what is being studied: "...transduced iTreg cells showed...", "...C/EBPβ-transduced human iTreg cells exhibited...". And for the final sentence: "...molecular target for enhancing the formation and stability of new Treg cells in inflammatory environments."
3) In the text describing Fig 5G, point out that transduced iTreg were sorted to remove non-transduced cells.

2nd Revision - authors' response | 20 September 2018

Summary of the changes in the new manuscript:

1. Abstract is edited following suggestions made by Editor and Reviewer #2
2. Modifications of statistics in the manuscript file with track changes have been checked and revised.
3. Statistical analysis for Fig. EV3B is added.
4. Scale bars in Fig. 2E and Fig. 5H are provided.
5. A "Table of contents" and page number are added to Appendix file.
6. Reference type is reformatted according to the EMBO reports style.
7. Synopsis is provided.
8. The range of y-axis in Fig. 1 is changed following Reviewer #2's suggestions.

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Corresponding Author Name: Rho H. Seong
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-45995V2

## Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

### A- Figures

#### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

#### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

### USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun
http://datadryad.org
http://figshare.com
http://www.ncbi.nlm.nih.gov/gap
http://www.ebi.ac.uk/ega
http://biomodels.net/
http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

### B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

| Question | Answer |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | No statistical methods were used to predetermine sample size. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | The statistical analyses were performed based on the experimental results obtained from more than three biological replicates and the statement about sample size estimate were called out in Figure legends. |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | N/A |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | We have used an unbiased approach when allocating animals or samples to treatment. |
| For animal studies, include a statement about randomization even if no randomization was used. | No randomization was used. |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | Yes, the clinical scoring of EAE mice was performed in double-blinded manner. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | See above |
| 5. For every figure, are statistical tests justified as appropriate? | Yes, the statistical tests used are stated in the figure legends. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Yes, the data meet the assumptions of the tests. |
| Is there an estimate of variation within each group of data? | Yes. |
| Is the variance similar between the groups that are being statistically compared? | Yes. |

### C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | It is described in Materials and Methods section. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | Jurkat cells were negative for mycoplasma contamination. |

* for all hyperlinks, please see the table at the top right of the document

### D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory. Foxp3EGFP mice and 2D2 TCR-transgenic mice were kindly provided by T. A. Chatila (the University of California at Los Angeles) and Dong-Sup Lee (Seoul National University), respectively. All mice were bred and maintained in specific pathogen-free barrier facilities at Seoul National University and were used according to protocols approved by the Institutional Animal Care and Use Committees (IACUC) of Seoul National University. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | N/A |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | Confirmed. |

### E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | Experiments involving human blood were approved by the Institutional Review Board at Seoul National University (SNUIRB No. 1502 / 001-013). |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | Human adult blood samples were anonymously provided by the Blood Center of the Korean Red Cross, Seoul, under the approval of the Institutional Review Board with consent for research use |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | N/A |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | N/A |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | N/A |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | N/A |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | N/A |

### F- Data Accessibility

| | |
|---|---|
| 18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | N/A |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | N/A |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | N/A |
| 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | N/A |

### G- Dual use research of concern

| | |
|---|---|
| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | N/A |