

The transcriptional regulator Sin3A balances IL-17A and Foxp3 expression in primary CD4 T cells

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Dear Dr. Mondino,

Thank you for the transfer of your research manuscript to EMBO reports. I have now received the 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, the referees think that these findings are of interest. However, all referees have several comments, concerns, and suggestions, indicating that revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here. As you will see, referee #1 has also novelty issues. Please address this in detail in your rebuttal letter.

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 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. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on $n=2$ (the authors are then asked to present scatter plots or provide more data points).

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Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843>)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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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.

Please use this link to submit your revision: <https://embor.msubmit.net/cgi-bin/main.plex>

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The major observation described in the manuscript by Perucho, et al. are that the transcriptional regulator, mSin3A, controls the balance between IL-17 producing Th17 cells and FoxP3 expressing T regulatory cells. The significance of these observations is that they outline a potential role for mSin3A in controlling the switch between these two mature CD4⁺ T cell subsets. Although this is of considerable interest, particularly to the immunology community, there is already a great deal known about the role of transcription factors, chromatin modifications and epigenetic mechanisms in the lineage commitment of T cell subsets.

More specific to this manuscript, there is already a significant amount of information known about the role of Sin3A in T cells, and the authors have committed a portion of this paper reconfirming previously published studies. For example, previously published studies have clearly shown an important role for Sin3A in T cell development. In the first sentence of the discussion, the authors claim that they have "uncovered its critical role in CD4⁺ T cell progenitors." However, the importance of Sin3A in T cell progenitors is well known.

In addition, the idea that Sin3 gene deletion in thymocytes halts T cell development is not a new finding as Cowley et al previously showed that expression of Sin3A in double positive thymocytes is essential for their survival and CD8 T cell development. *Mol Cell Biol.* 2005 Aug; 25(16): 6990-7004. Similarly, loss of the histone deacetylases 1 and 2 (HDAC1/2), which regulates chromatin structure as the catalytic core of the Sin3A, NuRD and CoREST co-repressor complexes, also leads to the disruption of T cell development. *Blood* (2013) 121 (8): 1335-1344.

Moreover, it is already also known that Sin3A directly interacts with STAT3 and promotes STAT3 deacetylation. Sin3A gene silencing leads to prolonged nuclear accumulation of STAT3, and recruitment to STAT3 regulated promoters (Icardi, et al. *PNAS* 2012 109(30) 12058-12063). Given the latter, it is somewhat intuitive that the loss of mSin3A would impact Th17 development, given the documented role of STAT3 in this process.

Therefore, given the availability of this information, it would be beneficial to know how the authors think the new information provided in the current manuscript further advances our understanding of the role of Sin3A in CD4 T cell differentiation and function. Some specific questions that should be addressed are below:

1. The data in Figure 1, demonstrating that there is a role for Sin3A in thymocyte development is not new. Thus, if the principal observation here that the function of Sin3A in mature T cells is more specific to differentiation and function rather than viability of progenitors, how can this be more clearly stated in the summary and how can the authors more appropriately reflect their observations in these conditional deletion models with the previously published work?
2. In addition, what are the mechanisms underlying the relative increase in IL-2 and FOXP3 expressing T cells? Said another way, what specific regulatory complexes are affected by the deficiency of Sin3A and how do they impact this lineage determination. The authors note a presumed 'causal link' because the disruption of IL-2 or Foxp3 partially restores IL-17 expressing T cells. Why? This is somewhat circumstantial and in no way confirmatory.
3. Mechanistically, the Sin3A transcriptional repressor complex is an obligatory partner of STAT3 on the promoters of the repressed genes, and STAT3 is known to be a repressor of Foxp3. So, it would be intuitive that the absence of Sin3A will lead to a loss of STAT3 at the Foxp3 promoter and enhanced Foxp3 expression. On the contrary, it is also known that STAT3 is an activator of IL-17 gene expression, and that disruption of STAT3 abrogates IL-17 expression. So, why then is there a preferential expansion of FOXP3 Treg and a relative deficiency of IL-17 expressing CD4⁺ T cells when the loss of Sin3A leads to increased nuclear STAT3? Are there differences in promoter occupancy by STAT3 in the absence of Sin3A in mature CD4⁺ T cells?
4. The authors suggest that Sin3A is 'balancing' or 'fine tuning' Th17 vs FoxP3⁺ Treg production. But how does mSin3A accomplish this? The model in Figure 8 is highly speculative and so much of it centers around the relative differences of STAT3 activity in Th17 and Foxp3 Treg cells in the absence of Sin3A. However, there are no analyses of STAT3 promoter occupancy to provide any direct evidence of a shift in the activity the transcriptional activity of STAT3 in the absence of Sin3A in mature CD4 T cells. Have the authors attempted to assess this, and would this not be an important analysis to include known the critical role of STAT3 in this process?
5. The acquisition of a mTORC signature is proposed, but again, a demonstrated mechanism for how the loss of Sin3A drives that signature is not provided. Much of the data are descriptive in nature, providing a correlation between Sin3A gene expression and phenotype but no mechanistic underpinning demonstrated. Moreover, these studies utilize CD4⁺ cells exposed to Tamoxifen, which is known to activate mTOR signaling and to increase the number of splenic Treg in mice. Furthermore, estrogen receptor signaling is known to affect Treg development/differentiation; see Goodman et al *PNAS* 2020; (<https://www.pnas.org/doi/10.1073/pnas.20022661170>). How might this influence the results of studies in which you are using 4OH Tamoxifen to induce conditional deletion of Sin3A?
6. In total, the studies described contribute more evidence that Sin3A is an important regulator of T cell fate and function. The data do reveal a distinct role for this multifunctional Sin3A repressor complex in mature CD4 T cells. However, the significance of these findings would be strengthened by the inclusion of in vivo model systems that reveal the context in which this function might be critical and by mechanistic studies that would provide insight into how the absence of Sin3A alters the abundance of Treg and Th17 cells. The latter could be quite context dependent and thus the pursuit of in vivo data would add value and impact to the observations provided. Otherwise, the information provided is somewhat descriptive and provides only a modest advancement of our understanding of the role of Sin3A in T cells.

Referee #2:

In this manuscript, Perucho et al investigates the effect of Sin3A (an epigenetic regulator) deficiency in Th17 CD4 cell differentiation using Sin3A f/f TAM-inducible Cre mice where they can disrupt Sin3A expression in mature CD4 cells without affecting T cell development. The study shows that Sin3A deficiency causes an impaired IL-17 production by Th17 cells, but increased Stat3 activation (and IL-21 production). They also show that the reduced IL-17 production is associated to an increase in FoxP3 expression. Overall, this is an interesting study showing for the first time the role of Sin3A in Th17 cell differentiation. There are a few concerns and questions that the authors could easily address with some more experiments.

Specific points:

1. Considering that tamoxifen (TAM) is an antagonist of estrogen and that estrogen is another transcriptional regulator of different genes, the authors should include some controls comparing Sin3A f/+ mice or +/+ mice with f/f mice all treated with TAM. Right now, most of the figures show data with NT (no TAM) and TAM, but the other control is essential as well. They do not need to repeat all the studies, but at least they should show the effect of TAM on IL-17, IFN γ , IL-22, IL-21 production as well as FoxP3 expression in f/+ mice or +/+ mice relative to NT f/+ or +/+ mice.
2. The production of IL-21 by Th17 cells is exclusively driven by IL-6 in the Th17 conditions (there are no many IL-17+ IL21+ cells in Th17 cells), and IL-6 by itself in the absence of TGF β drives the expression of IL-21. In addition, IL-21 is the main cytokine produced by Tfh cells (differentiation promoted by IL-6). As the authors describe, IL-21 expression is absolutely dependent on Stat3. Considering their data showing that TAM treatment of Sin3A f/f cells increases Stat3 and phospho-Stat3 levels, as well as IL-21, the authors should also examine the effect of Sin3A deficiency in cells maintained only with IL-6 (Tfh like cells) and further examine the regulation of Stat3 and IL-21. It is quite possible that the main effect of Sin3A is to be a negative regulator of Stat3 and IL-21.
3. The data with the P60 peptide acting as an inhibitor of FoxP3 (Fig. 6D) do not support the conclusions that inhibition of FoxP3 (P60) rescues IL-17 production in Sin3A deficient cells, because the ratio of NT/TAM for IL-17+ is basically the same between the non P60 treated and non-treated. The interpretation would be that the decreased in IL-17 producing cells is independent on FoxP3 and these conclusions are totally OK with their model. The interesting question, not addressed in the manuscript, is whether the increased FoxP3 cells in TAM Sin3A cells is due to the decreased in IL-17. The authors should try to rescue the effect by adding IL-17 or IL-22 (also reduced).

Referee #3:

The study by Perucho et al. investigates the role of the transcriptional regulator Sin3A in the development and differentiation of CD4+ T cells. To this end, the authors have generated transgenic mice with a specific deletion of Sin3A from the DP stage of thymic development by crossing Sin3A^{flox/flox} with CD4-Cre, CD4-CreER and Rosa26-CreERT2 mice. The authors show that Sin3A arrests the development of single-positive thymocytes. To overcome this developmental arrest and analyze the role of Sin3A in mature T cells, they used a Tamoxifen-induced deletion and claim that post-thymic inactivation of Sin3A alters proper differentiation of Th17 cells. Overall, the results observed with this new mouse model are new.

Concerns:

-How do the authors reconcile their results with those of Cowley et al. that used a Lck-Cre driven deletion of Sin3A at the DN stage ?

-In Fig. 1: In addition to the frequencies, it would be informative to show absolute numbers of thymocyte subsets.

At which developmental stage does Sin3A affect thymocytes? in positively-selected DP thymocytes, semi-mature SP, mature SP thymocytes?

Does Sin3 A affect the survival and/or proliferation of thymocytes in Sin3A^{flox/flox} with CD4-Cre mice?

-Does Sin3 A affect the development of Foxp3+ Treg in the thymus?

-Frequencies are lacking in the dot-plots depicted in Fig. 1H.

-What is the effect of the in vivo conditional deletion of Sin3A (in Sin3A^{flox/flox} x Rosa26-CreER mice treated with tamoxifen) in Th17 differentiation in inflammatory conditions?

-The experiments shown in Fig. S4 with the OTII system need to be repeated with APC loaded or not with OVA peptide to

interpret the role of Sin3A.

-MFI are lacking in Fig.2A and Fig.2B ?

-Statistics are lacking in Fig S1B and Fig 7F.

-The effect shown in Fig 7A on IL-2 production seems to be modest.

- What is the fraction of Sin3A deficient cells that preserves IL17A expression? Frequencies are lacking in the dot-plots depicted in Fig.S5B.

-The quantification of pSTAT5 shown in Fig.S7B is not convincing.

- By RNA-seq experiments, the authors found 1139 genes up-regulated and 2282 genes down-regulated. These data appear to be underutilized. They identified that only the mTORC1 is affected by the loss of Sin3A. To corroborate this conclusion, it would be useful to show a heatmap. Are other processes/signaling pathways affected?

-Minor typos

Point-by-point reply to EMBOR-2022-55326

We thank the Editor and the reviewers for the interest shown in our work and for the time dedicated to improving it. We have taken all reviewers' concerns into serious consideration and addressed them to the best of our knowledge and according to previously available and newly generated data.

Overall, the revision led to the inclusion of new data depicted in 6 new Figures, 1 within the Main Section and 5 in the Supplementary one. These data further clarify the mechanisms subtending the loss of Sin3A over the course of Th-17 differentiation.

As you will see from the point-by-point reply, we could not perform the requested *in vivo* experiment, due to defective survival of mature T cells upon Sin3A inactivation. This was not fully predictable, and yet we had to resign to the use of Sin3A deficient CD4 T cell *in vitro*. Nevertheless, our study is original and uncovers a previously unappreciated role for Sin3A in dictating Th17 cell differentiation, via the balancing of IL-2, mTOR, STAT3, Foxp3 and ROR γ t.

The enclosed point-by-point reply has been prepared to respond to the reviewers suggestion/criticism.

Referee #1:

The major observation described in the manuscript by Perucho, et al. are that the transcriptional regulator, mSin3A, controls the balance between IL-17 producing Th17 cells and FoxP3 expressing T regulatory cells. The significance of these observations is that they outline a potential role for mSin3A in controlling the switch between these two mature CD4+ T cell subsets. Although this is of considerable interest, particularly to the immunology community, there is already a great deal known about the role of transcription factors, chromatin modifications and epigenetic mechanisms in the lineage commitment of T cell subsets.

More specific to this manuscript, there is already a significant amount of information known about the role of Sin3A in T cells, and the authors have committed a portion of this paper reconfirming previously published studies. For example, previously published studies have clearly shown an important role for Sin3A in T cell development. In the first sentence of the discussion, the authors claim that they have "uncovered its critical role in CD4+ T cell progenitors." However, the importance of Sin3A in T cell progenitors is well known.

In addition, the idea that Sin3 gene deletion in thymocytes halts T cell development is not a new finding as Cowley et al previously showed that expression of Sin3A in double positive thymocytes is essential for their survival and CD8 T cell development. Mol Cell Biol. 2005 Aug; 25(16): 6990-7004. Similarly, loss of the histone deacetylases 1 and 2 (HDAC1/2), which regulates chromatin structure as the catalytic core of the Sin3A, NuRD and CoREST co-repressor complexes, also leads to the disruption of T cell development. Blood (2013) 121 (8): 1335-1344.

Moreover, it is already also known that Sin3A directly interacts with STAT3 and promotes STAT3 deacetylation. Sin3A gene silencing leads to prolonged nuclear accumulation of STAT3, and recruitment to

STAT3 regulated promoters (Icardi, et al. PNAS 2012 109(30) 12058-12063). Given the latter, it is somewhat intuitive that the loss of mSin3A would impact Th17 development, given the documented role of STAT3 in this process.

Therefore, given the availability of this information, it would be beneficial to know how the authors think the new information provided in the current manuscript further advances our understanding of the role of Sin3A in CD4 T cell differentiation and function.

We thank the reviewer for his/her comment acknowledging our study of “considerable interest, particularly to the immunology community”. We agree that *“there is already a great deal known about the role of transcription factors, chromatin modifications and epigenetic mechanisms in the lineage commitment of T cell subsets”*. Yet, we respectfully point out that a role for Sin3A in shaping CD4⁺ Th differentiation has never been reported.

In the following response to **Reviewer’s 1** specific questions we clarified the novelty of our work, attempted to resolve possible concerns, and acknowledged current limitations of our study.

Some specific questions that should be addressed are below:

1. The data in Figure 1, demonstrating that there is a role for Sin3A in thymocyte development is not new. Thus, if the principal observation here that the function of Sin3A in mature T cells is more specific to differentiation and function rather than viability of progenitors, how can this be more clearly stated in the summary and how can the authors more appropriately reflect their observations in these conditional deletion models with the previously published work?

The reviewer is correct in pointing out that a role for Sin3A in thymocyte development was previously reported (Cowley et al. 2005). However, it is worth underlying that the mentioned study adopted Lck-Cre mice, with Sin3A deletion at the DN thymocyte stage, and mainly described a phenotype for CD8 precursors. We instead choose CD4-Cre deleter strains, in the hope that deletion of Sin3A past pre-TCR β selection (DN3 stage) would allow thymocyte maturation. In the manuscript we report that deleting Sin3A at the CD4⁺CD8⁺ DP stage halts positive selection, and proper thymocyte development.

To investigate the contribution of Sin3A to mature CD4⁺ T cells differentiation, we then took advantage of T cells derived from CD4-CreER^{T2}/+ Sin3A F/F mice and tamoxifen-inducible deletion. This allowed to delete Sin3A in mature T cells, and investigate effects on T cell differentiation.

We have revised the summary and the text to better underline these aspects, making specific reference to progenitors and mature CD4 T cells (page 11-12).

2. In addition, what are the mechanisms underlying the relative increase in IL-2 and FOXP3 expressing T cells? Said another way, what specific regulatory complexes are affected by the deficiency of Sin3A and how do they impact this lineage determination. The authors note a presumed 'causal link' because the disruption of IL-2 or Foxp3 partially restores IL-17 expressing T cells. Why? This is somewhat circumstantial and in no way confirmatory.

We thank the reviewer for stimulating further discussion. We originally presumed a causal link because neutralizing IL-2 reduced FoxP3⁺ cells and rescued to some extent IL-17. The reviewer is correct in pointing out that these events could also correlate, rather than being causally linked. As anti-IL-2 only caused a partial rescue, we looked for additional mechanisms. We found that cells lacking Sin3A have increased ROR γ t cytoplasmic levels. We revised the text to clarify that IL-2 and FoxP3 might contribute to the inhibition of il17, but that additional mechanisms are in place (page 18, 23).

3. Mechanistically, the Sin3a transcriptional repressor complex is an obligatory partner of STAT3 on the promoters of the repressed genes, and STAT3 is known to be a repressor of Foxp3. So, it would be intuitive that the absence of Sin3A will lead to a loss of STAT3 at the Foxp3 promoter and enhanced Foxp3 expression. On the contrary, it is also known that STAT3 is an activator of IL-17 gene expression, and that disruption of STAT3 abrogates IL-17 expression. So, why then is there a preferential expansion of FOXP3 Treg and a relative deficiency of IL-17 expressing CD4⁺ T cells when the loss of Sin3A leads to increased nuclear STAT3? Are there differences in promoter occupancy by STAT3 in the absence of Sin3A in mature CD4⁺ T cells?

The description of the Sin3a transcriptional repressor complex as an obligatory partner of STAT3, able to control its acetylation status, was first provided in Hek293T and MCF7 cells by Laura Icardi (second author of the current manuscript, when she was a PhD student in Jan Tavernier group; Icardi, 2012). More recently Laura described a similar function in NPM-ALK⁺ anaplastic large-cell lymphoma (Gambi et al, 2019). To the best of our knowledge, whether Sin3A is an obligatory partner of STAT3 in primary T cells, and/or contribute to T cell differentiation remains to be defined.

Our study is to first to address the contribution of Sin3A to primary mature Th17 cell differentiation, and its ability to control not only STAT3, but also other factors critical for T cell differentiation. We did consider exploring STAT3 promoter occupancy in Sin3A sufficient and deficient cells. However, the finding that Sin3A inactivation imposes an mTOR signature and also hinders ROR γ t, suggested that also STAT3-independent events are likely relevant. Thus, while such analyses would surely help understanding STAT3/Sin3A regulation in T cells, they might not necessarily clarify the observed phenotype. Dedicated experiments are now being planned. We discussed their importance in the revised manuscript (page 23).

4. The authors suggest that Sin3A is 'balancing' or 'fine tuning' Th17 vs FoxP3⁺ Treg production. But how does mSin3A accomplish this? The model in Figure 8 is highly speculative and so much of it centers around the relative differences of STAT3 activity in Th17 and Foxp3 Treg cells in the absence of Sin3A. However, there are no analyses of STAT3 promoter occupancy to provide any direct evidence of a shift in the activity the transcriptional activity of STAT3 in the absence of Sin3A in mature CD4 T cells. Have the authors attempted to assess this, and would this not be an important analysis to include known the critical role of STAT3 in this process?

In the summary figure we meant to integrate our results with available literature, and generate a working model. We have revised the model to better highlight the relative contribution of both STAT3 and ROR γ t to the observed phenotype (Supplementary Fig. 12B). We also outlined current gaps in knowledge that await further characterization to fully understand the role of STAT3 (and ROR γ t) to Th17 differentiation (revised discussion).

5. The acquisition of a mTORC signature is proposed, but again, a demonstrated mechanism for how the loss of Sin3A drives that signature is not provided. Much of the data are descriptive in nature, providing a correlation between Sin3A gene expression and phenotype but no mechanistic underpinning demonstrated.

Data indicated that deletion of Sin3A had opposite effects in *il2* and *il17a* expression and that blocking IL-2 partially rescued IL-17+ cells. This prompted us to suggest these events to be mechanistically linked. IL-2 can also activate mTOR, and in line with this, we found that genes belonging to mTOR signaling are upregulated upon Sin3A deletion. Whether, IL-2 is directly and uniquely responsible for such gene signature or additional Sin3A-controlled events also participate (more likely), remains to be determined. Nevertheless the reviewer makes a fair point. We carefully revised the text to clarify that some of the described events remains correlative in nature (revised discussion).

Moreover, these studies utilize CD4+ cells exposed to Tamoxifen, which is known to activate mTOR signaling and to increase the number of splenic Treg in mice. Furthermore, estrogen receptor signaling is known to affect Treg development/differentiation; see Goodman et al PNAS 2020; (<https://www.pnas.org/doi/10.1073/pnas.20022661170>). How might this influence the results of studies in which you are using 4OH Tamoxifen to induce conditional deletion of Sin3A?

We apologize for not having included all the controls in the original manuscript. Original Supp. Fig. 4, now Supp. Fig.6 depicted control and 4OH Tamoxifen treated cultures of Cre+ F/+ (Sin3A sufficient) or Cre+ F/F (Sin3A deficient cells) to simultaneously verify possible Cre and tamoxifen-specific effects over the course of cell activation and proliferation. We have now added new data to confirm that 4OH Tamoxifen does not affect IL-17, IFN- γ , IL-2, or FoxP3 levels (Supp. Fig. 9 and page 17). Together our data indicate that 4OH Tamoxifen per se does not has an effect on the studied phenotypes.

6. In total, the studies described contribute more evidence that Sin3A is an important regulator of T cell fate and function. The data do reveal a distinct role for this multifunctional Sin3A repressor complex in mature CD4 T cells. However, the significance of these findings would be strengthened by the inclusion of in vivo model systems that reveal the context in which this function might be critical and by mechanistic studies that would provide insight into how the absence of Sin3A alters the abundance of Treg and Th17 cells. The latter could be quite context dependent and thus the pursuit of in vivo data would add value and impact to the observations provided. Otherwise, the information provided is somewhat descriptive and provides only a modest advancement of our understanding of the role of Sin3A in T cells.

We thank the reviewer for acknowledging that our data support the notion that Sin3A is critical to T cell fate and function, and play a distinct role in mature T cells.

We agree that the inclusion of *in vivo* analysis would have contributed to the significance of the findings. We crossed Sin3A F/F mice to the CD4-Cre or the CD4-Cre CreER^{T2} deleter strains exactly with this purpose in mind. Unfortunately we found that deleting Sin3A in double-positive thymocyte progenitors or in post-thymic mature T cells hinder their survival *in vivo*. We added more data (revised Supp. Fig.3C-D), to further clarify this point. Because of this, we had to restrict our study to *in vitro* analyses.

Referee #2:

In this manuscript, Perucho et al investigates the effect of Sin3A (an epigenetic regulator) deficiency in Th17 CD4 cell differentiation using Sin3A f/f TAM-inducible Cre mice where they can disrupt Sin3A expression in mature CD4 cells without affecting T cell development. The study shows that Sin3A deficiency causes an impaired IL-17 production by Th17 cells, but increased Stat3 activation (and IL-21 production). They also show that the reduced IL-17 production is associated to an increase in FoxP3 expression. Overall, this is an interesting study showing for the first time the role of Sin3A in Th17 cell differentiation. There are a few concerns and questions that the authors could easily address with some more experiments.

We thank the reviewer for having pointed out the novelty of our work and for the insightful suggestions.

Specific points:

1. Considering that tamoxifen (TAM) is an antagonist of estrogen and that estrogen is another transcriptional regulator of different genes, the authors should include some controls comparing Sin3A f/+ mice or +/+ mice with f/f mice all treated with TAM. Right now, most of the figures show data with NT (no TAM) and TAM, but the other control is essential as well. They do not need to repeat all the studies, but at least they should show the effect of TAM on IL-17, IFN γ , IL-22, IL-21 production as well as FoxP3 expression in f/+ mice or +/+ mice relative to NT f/+ or +/+ mice.

We apologize for not having included such data in the original manuscript. Original Supp. Fig. 4, now Supp. Fig.6, depicted the requested controls, i.e. Cre⁺ F/+ (Sin3A sufficient) or Cre⁺ F/F (Sin3A deficient cells) in TAM-treated cultures, but only compared cell activation and proliferation. We have now added new data showing that TAM does not have an effect on IL-17, IFN γ , IL-2, or FoxP3 (New Supp. Fig. 9, page 17).

2. The production of IL-21 by Th17 cells is exclusively driven by IL-6 in the Th17 conditions (there are no many IL-17⁺ IL21⁺ cells in Th17 cells), and IL-6 by itself in the absence of TGF β drives the expression of IL-21. In addition, IL-21 is the main cytokine produced by Tfh cells (differentiation promoted by IL-6). As the authors describe, IL-21 expression is absolutely dependent on Stat3. Considering their data showing that TAM treatment of Sin3A f/f cells increases Stat3 and phospho-Stat3 levels, as well as IL-21, the authors should also

examine the effect of Sin3A deficiency in cells maintained only with IL-6 (Tfh like cells) and further examine the regulation of Stat3 and IL-21. It is quite possible that the main effect of Sin3A is to be a negative regulator of Stat3 and IL-21.

We performed the experiment suggested by the reviewer, and maintained CD4⁺ T cells in Tfh like conditions, or in Th17-noTGFb conditions, or in IL-6 only, or in IL-6+IL-21 in different amounts). We repetitively failed to detect IL-21, and only observed partial effects on IL-21 mRNA in Sin3A sufficient cells. We agree that Sin3A can act as negative regulator of STAT3, possibly downstream IL-6. The finding that STAT3 phosphorylation and nuclear abundance are increased in the absence of Sin3A, and that *il21* is upregulated support this possibility. We will explore different cell systems to address the reviewer suggestion. To raise the interest in this possibility, we discussed it in the revised discussion.

3. The data with the P60 peptide acting as an inhibitor of FoxP3 (Fig. 6D) do not support the conclusions that inhibition of FoxP3 (P60) rescues IL-17 production in Sin3A deficient cells, because the ratio of NT/TAM for of IL-17⁺ is basically the same between the non P60 treated and non-treated.

The reviewer is indeed correct in stating that when considering the NT/TAM ratio the FOXP3 inhibitor (P60) equally affects Sin3A sufficient and deficient cells. We apologize for not having realized this before. Keeping this in mind, these data should be interpreted as FOXP3 inhibition to rescue IL17A levels, independently of Sin3A. We have revised the text to clarify this point (page 18).

The interpretation would be that the decreased in IL-17 producing cells is independent on FoxP3 and these conclusions are totally OK with their model. The interesting question, not addressed in the manuscript, is whether the increased FoxP3 cells in TAM Sin3A cells is due to the decreased in IL-17. The authors should try to rescue the effect by adding IL-17 or IL-22 (also reduced).

We performed the suggested experiments and supplemented Sin3A sufficient and deficient cell cultures with IL-17 or IL-22. Experiments revealed some variability. Results depicted in Figure R1 for Reviewer perusal only, depict one of three independent determination. Trends were confirmed in a second set of data, but not in a third one. Data indicate that exogenous IL-17 or IL-22 lower IL-17A in Sin3A sufficient cells, while they moderately increase IL-17⁺ cells among Sin3A deficient ones. In the case of Foxp3, IL-17 or IL-22 increased FoxP3⁺ cells among both Sin3A sufficient and deficient cells.

As the prediction was that IL-17 and IL-22 would have lowered FOXP3 levels in Sin3A deficient cells, we do not feel that including these data would help clarity of the manuscript. We thank the reviewer for the suggestion. We did discuss the suggested interpretation in the revised discussion (page 23).

Referee #3:

The study by Perucho et al. investigates the role of the transcriptional regulator Sin3A in the development and differentiation of CD4+ T cells. To this end, the authors have generated transgenic mice with a specific deletion of Sin3A from the DP stage of thymic development by crossing Sin3A^{flox/flox} with CD4-Cre, CD4-CreER and Rosa26-CreERT2 mice.

The authors show that Sin3A arrests the development of single-positive thymocytes. To overcome this developmental arrest and analyze the role of Sin3A in mature T cells, they used a Tamoxifen-induced deletion and claim that post-thymic inactivation of Sin3A alters proper differentiation of Th17 cells. Overall, the results observed with this new mouse model are new.

We thank the reviewer for having acknowledged the novelty of our work.

Concerns:

-How do the authors reconcile their results with those of Cowley et al. that used a Lck-Cre driven deletion of Sin3A at the DN stage ?

We apologize for not having more clearly compared our findings with those previously reported by Cowley and co-authors. The reviewer is correct in pointing out that these authors adopted the Lck-Cre driven deletion of Sin3A at the DN stage. We instead adopted a CD4-Cre deleter strain to see whether this could rescue mature T cell development, and later a CD4-Ert2-Cre delete strain to study mature T cells.

We have now revised the manuscript to underline differences in the timing of Sin3A deletion (at the DN stage in their report, at the DP stage and in mature post-thymic CD4 T cells in our manuscript) and resulting phenotypes (page 11, 21), and most importantly the fact that we investigated the effects of Sin3A ablation in mature T cells in Th17 conditions. This latter question was not previously investigated.

-In Fig. 1: In addition to the frequencies, it would be informative to show absolute numbers of thymocyte subsets.

We thank the reviewer for pointing out this. The Figure has now been revised to include the missing frequencies. In addition, Total numbers of thymocyte and thymocyte subsets are now depicted in New Supplementary Figure 1.

-At which developmental stage does Sin3A affect thymocytes? in positively-selected DP thymocytes, semi-mature SP, mature SP thymocytes?

We have investigated this question performing flow cytometry analysis. Data are depicted in New Supplementary Figure 1 and Supplementary Figure 2. Results indicate that more mature SP cells are less represented in the thymus of CD4-Cre⁺ mice, indicating a failure in positive selecting. Given the higher

propensity of these cells to undergo apoptosis, we suggest reduced survival best explain peripheral lymphopenia.

-Does Sin3 A affect the survival and/or proliferation of thymocytes in Sin3A^{flox/flox} with CD4-Cre mice?

As replied above, we investigated survival by measuring apoptosis by flow cytometry ex vivo. We found higher fraction of Annexin V⁺ cells in the thymus of CD4-Cre⁺ Sin3A F/F mice compared to controls, supporting a defect in survival. Data have been added to the revised manuscript (New Supplementary Fig. 1).

-Does Sin3 A affect the development of Foxp3⁺ Treg in the thymus?

We did investigate the issue, and the results indicate that Foxp3⁺ cells are detectable among SPCD4⁺ thymocytes, although to a lower frequency in CD4-Cre⁺ Sin3A F/F mice compared to controls. We think this mostly relates to defective survival of Sin3A deficient cells undergoing positive selection. Data are reported in Supp. Fig. 1C and commented in the revised manuscript (page 11-12).

To further analyze this point we also analyzed peripheral CD4⁺ FoxP3⁺ Treg representation. Data indicate that CD4 T cells are less represented in the blood of CD4-Cre⁺ Sin3A F/F mice, compared to controls. As in the case of secondary lymphoid organs (Figure2), both Sin3⁺ and Sin3A⁻ cells could be detected, and while the former revealed an effector memory phenotype the latter proved mainly naïve. When compared within EM and Naïve T cells, the frequency of CD25⁺FoxP3⁺ cells was comparable. Thus, Sin3A affects also Treg development. This has been clarified in the manuscript (page 12).

-Frequencies are lacking in the dot-plots depicted in Fig. 1H.

We thank the reviewer for pointing out this. The Figure has now been revised to include the missing frequencies.

-What is the effect of the in vivo conditional deletion of Sin3A (in Sin3A^{flox/flox} x Rosa26-CreER mice treated with tamoxifen) in Th17 differentiation in inflammatory conditions?

We generated Sin3 F/F x CD4-Cre-ER^{T2} mice with the specific purpose of understanding the impact of Sin3A inactivation on mature T cells and over the course of inflammatory conditions. Unfortunately, as also stated in response to Reviewer 1, we found that deletion of Sin3A in mature T cells is not compatible with their in vivo survival.

-The experiments shown in Fig. S4 with the OTII system need to be repeated with APC loaded or not with OVA peptide to interpret the role of Sin3A.

We apologize for lack of clarity. Data depicted in Original Supp. Fig. 4, now Supp. Fig. 6 were obtained by stimulating the cells with the OVA peptide₃₂₃₋₃₃₉ (1.25 μ M). We revised the text to better describe the experiment.

-MFI are lacking in Fig.2A and Fig.2B ?

We have revised the Figure to report the MFI of depicted data.

-Statistics are lacking in Fig S1B and Fig 7F.

We have revised the Figures to include statistics.

-The effect shown in Fig 7A on IL-2 production seems to be modest.

Th17 skewed cells typically produce less IL-2 when compared to naïve/Th1, and IL-2 normally inhibits Th17 cell differentiation (references within the manuscript). Thus, a low frequency could be expected. We agree that in some experiment the IL-2⁺ cell frequency was indeed somehow low (5%), and yet differences among Sin3A sufficient and deficient cells over independent determination revealed highly significant. In spite of this, we think IL-2 plays a role, but it is not the only event responsible for defective IL-17A. We have highlighted these points in the revised manuscript (revised discussion).

- What is the fraction of Sin3A deficient cells that preserves IL17A expression? Frequencies are lacking in the dot-plots depicted in Fig.S5B.

We have revised the Figure to report the frequency of IL-17⁺ cells among Sin3A sufficient and deficient cells. As noted by the reviewer a small fraction Sin3A deficient cells preserves IL-17A expression. Whether this is due to residual Sin3A expression at the time of activation, or to Sin3A independent events remains to be understood.

-The quantification of pSTAT5 shown in Fig.S7B is not convincing.

To better explain the result, we revised the figure to show that the increase of STAT5 was reproducibly found in each individual experiment. We also were skeptical, and yet differences were reproducible and of statistical significance. As STAT5 phosphorylation is (also) controlled by IL-2, variability might also rely to relative IL-2 abundance in the cultures. We discussed this point in the revised manuscript.

- By RNA-seq experiments, the authors found 1139 genes up-regulated and 2282 genes down-regulated. These data appear to be underutilized. They identified that only the mTORC1 is affected by the loss of Sin3A. To corroborate this conclusion, it would be useful to show a heatmap. Are other processes/signaling pathways affected?

We thank the reviewer for the remarks. We provide the requested heatmap in new Supp. Fig. 7A, which reports the top 35 up and downregulated genes among the 181 of the mTORC1 signature.

We also re-analyzed the data and confirmed that the mTORC1 signature was the only significantly changed by the loss of Sin3A according to GSEA analyses.

Nevertheless, to deepen the analysis we also adopted a different approach, and individually considered only genes that were upregulated ($5 > \log_2FC > 1$) or only downregulated ($-1 > \log_2FC > -7$) with an adjusted p value < 0.01 to generate a gene list to be compared to available data bases adopting the EnrichR engine. When using the down-regulated ones, we found them to belong to the cytokine receptor interaction (adj p-value < 0.002) and IL-17 signaling pathway (adj p-value < 0.02). When using the upregulated ones, we found them to belong to amino acid, vitamin and fatty acid metabolism ($0.001 < p\text{-value} < 0.05$). Data are reported in new Supp. Fig. 7B-C. These computational analyses appear in line with the upregulation of the mTORC1 signature and the inability of the cells to differentiate towards Th17 cells.

Furthermore, we evaluated the overlap of the identified gene lists with the ENCODE (transcription) plus ChEA (Chip-ex) integrated database (described by Lachmann, A. 2010; PMID: 20709693) to more directly analyze Sin3A-dependent transcriptional regulation. We found that down regulated genes overlapped with the Polycomb 2-regulated gene group, while the upregulated ones overlapped with gene groups controlled by myc/max family members (Supp. Fig. 7D-E). These further analyses, although they support the involvement of Sin3A in instructing a mTORC1 signature possibly critical to Th17 differentiation, suggest that Sin3A plays a broader than expected in T cell biology.

The text has been revised to report these novel analyses (page 15)

-Minor typos

We apologize, and have carefully revised the manuscript.

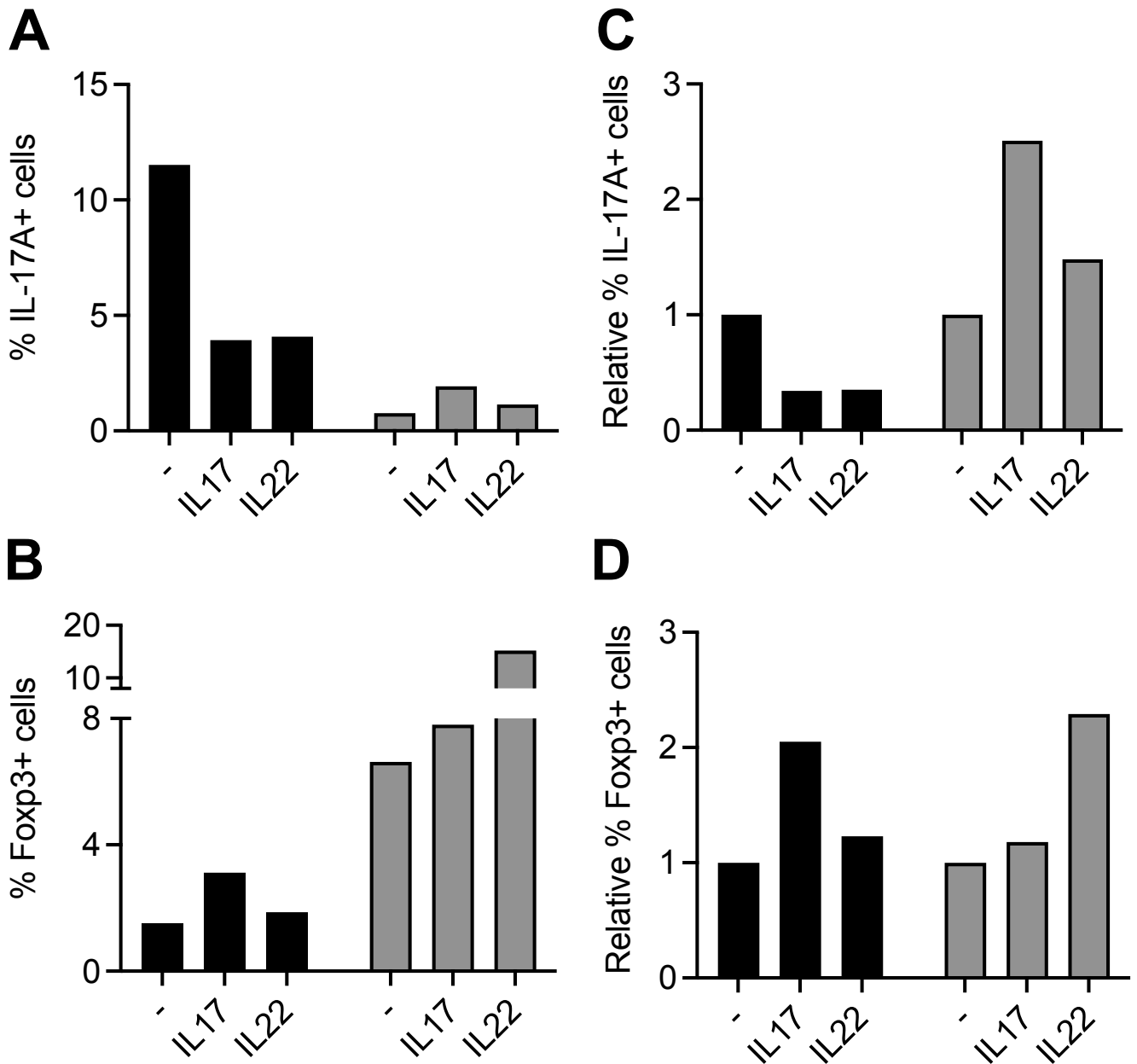


Figure R1. Effects of recombinant IL-17 or IL-22. Cells were cultured as depicted in Figure 2 and then left untreated or stimulated with PMA and Ionomycin for 4h. One of three experiments is depicted. A-B) Frequency of CD4+ IL-17 or FoxP3+ cells is depicted. C-D) Fold over untreated was calculated.

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Dr. Anna Mondino
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Include a statement about blinding even if no blinding was done.	Not Applicable	Blinding was not needed
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not Applicable	Normal distribution with equal variance was. Outliers were excluded when identified
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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of select agents and toxins (CDC): https://www.selectagents.gov/sat/list.htm .	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the authority granting approval and reference number for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., ICMJE, MIBBI, ARRIVE, PRISMA) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Materials and Methods
Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective data citations in the reference list .	Not Applicable	