THE EMBO JOURNAL

Batf Stabilizes Th17 Cell Development via Impaired Stat5 Recruitment of Ets1-Runx1 Complexes

Duy Pham, Daniel Silberger, Kim Nguyen, Min Gao, Casey Weaver, and Robin Hatton
DOI: 10.15252/embj.2021109803

Corresponding author(s): Robin Hatton (robinhatton@uabmc.edu) , Casey Weaver (cweaver@uab.edu

Review Timeline:

Submission Date:	24th Sep 21
Editorial Decision:	27th Oct 21
Revision Received:	16th Nov 22
Editorial Decision:	21st Dec 22
Revision Received:	13th Jan 23
Accepted:	19th Jan 23

Editor: Karin Dumstrei

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Weaver,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments, while the referees appreciate that the analysis they also find that the findings need to be extended to consider publication here. Should you be able to address the concerns raised by the referees then I would like to invite a revised version.

I think it would be helpful to discuss the referee points further before you embark on the revisions. We can do so via email or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

I thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further with you.

Please note that I have attached a document with helpful tips on how to prepare the revised version

sincerely,

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days. Please contact the editor if you need an extension. Click on the link below to submit the revision online before 25th Jan 2022:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

In this study, Pham et al examine the mechanisms by which Batf regulates CD4 helper cell differentiation and Th17 cell stabilization. The authors show that Batf deficiency results in upregulation of IFNg- and Foxp3-transcriptomic modules and reduction in expression of genes participating in the Th17 program (e.g. Il23r, Rorc, Maf, Il17a, Il17f and IL21). These findings were confirmed using siRNA and ectopic expression of Batf. In vivo, Batf deficiency was associated with impaired host defense against C. rodentium and aberrant helper T cell differentiation. The authors next employ Chip-seq for Batf and histone modifications (in WT and knockout cells) and ATACseq in in vitro differentiated Th17 cells. The authors show that Batf binds the Il2ra locus and its absence is associated with loss of repressive marks with increased expression in vitro and in vivo. This is associated with increased STAT5 phosphorylation and genomic binding, along with increased accessibility and deposition of H3K27Ac surrounding Th1 and Treg loci. STAT5 Chipseq in Batf deficient cells shows enrichment of Runx and Ets motifs and pharmacologic inhibition of Stat5 increased binding of Batf and STAT3 to the Il17 locus. In the context of their previous demonstration of instability of Th17 cells over time, the authors show that in the absence of IL-6 and TGFb, Batf expression declines over time with the enhancement of IL-2/Stat5 signals and recruitment of Ets1 and Runx1 to Th1 and Treg loci.

This manuscript tackles an important issue and provides interesting new mechanistic insights. In general, the manuscript is well-written and thoughtful and provides comprehensive and convincing data for the proposed mechanism. However, the authors should consider the following points:

It is unclear why the authors define STAT motifs as a half site and not a full palindrome. Do the authors believe that STATs are predominantly functioning as monomers partnered with Ets or Runx rather than as STAT dimers?

If feasible, the in vivo significance of the work might be enhanced by including ATACseq and transcriptomic data using cells

from Citrobacter infected Batf-deficient mice.

The gating on IL-22 producers is confusing; it is unclear whether all cells produce small amounts of IL-22 or the major peak represents non-producers; clarification is necessary. Based on ATACseq and Chip-seq data is the Il22 locus closed?

The Stat5 inhibitor used is an antipsychotic drug identified in a repurposing screen. It is unclear why this approach was used rather than STAT5 cKO mice or alternatively STAT5 siRNA; either would be useful to verify the results with pimozide.

The scale depicted in Figure 6 minimizes differences in STAT3 binding; the authors should be more circumspect in their conclusions.

Minor points/suggestions:

Global Batf deletion negatively impacts ILCs; the authors should consider whether phenotype seen in Figure 1 may also be result of impaired ILC3 function.

Given the impact of Batf on IL-2 signaling, might it be informative to show CFSE along with cytokine production?

Anti-IFNg antibody is included in Th17 cultures but given that Batf deficiency potently enhances IFNg production, the question arises whether dysregulation of helper cell programs may in part be due to IFNg. How confident are the authors in terms of the efficacy of blockade? Controls reinforcing this point could be useful.

In figure 1, it would likely be of interest to identify/label the most highly induced genes

What is the relative importance of IL-6 vs IL-23 on Batf expression; does IL-6 alone induce Batf and what if any, is the role of TGFb? Is the effect of TGFb principally related to inhibition of IFNg rather than promoting Batf expression?

Tbx21 is not altered by Batf deficiency; however, is the converse operative? Using public datasets does T-bet directly regulate Batf or is this largely mediated by Ifng?

The data provided in Figure 2 would be strengthened by quantifying and displaying overall differential accessibility.

Figure 7B: no cumulative data are provided relating to pStat5 levels. It is notable that the relatively high expression of pStat5 in Th17(R3) cells does not correlate with low level of Il2ra (Figure 7A). Some explanation is warranted

Figure 1O: cumulative data of percent IFNg+, IL-17a+, and CD25+ Foxp3+ cells in WT and Baft KO are not provided.

The titles for Figures 5 and 6 are identical. Better articulation of the take-home message would help. Also, no subheadings are used in the results section; subheadings would improve readability. More paragraph breaks would also be helpful, especially when the manuscript is in its published format.

Referee #2:

The study "Batf stabilizes the Th17 developmental program through impairment of Stat5-dependent recruitment of Ets-Runx1 complexes" by Pham addresses so far unstudied aspects alongside differentiation of T helper cells into Th17 cells previously reported to be pioneered by the AP1 transcription factor Batf together with IRF4.
This study was motivated by the observation that T helper cells deficient for Batf display a gene expression signature suggestive of a Th1 /Treg phenotype. Mechanistically the authors identify that T cells deficient for Batf express enhanced levels of IL-2ra/CD25 and argue that this condition may underlie the cells' increased permissiveness for IL-2/Stat5 signaling, especially fueling Th1 and Treg related gene loci. Mechanistically, enhanced Stat5 binding of Th1/Treg differentiation related loci in the absence of Batf was accompanied by increased Ets1 and Runx1 containing occupancies and Th1 /Treg related gene expression profiles. Blocking CD25 and Stat5 signaling directly reduced Th1/Treg prone gene signature of Batf deficient T cells cultured under Th17 conditions.

This study is giving interesting and so far not reported insight into the multi-faceted functionality of Batf driven T helper lineage specification. Addressing this important area is highly relevant but following concerns and suggestions may be addressed or followed up respectively to strengthen the significance and specificity of the results presented in that manuscript.

1. Re-analyzing microarray data from Ken Murphy's group (Fig. 1A/B) showing for the first time the critical role for Batf during Th17 differentiation the authors made the observation that Batf deficient T cells polarized under Th17 conditions display a Th1 like signature. Given the pioneering function of Batf in the context of gene regulation in T cells and the well- known observation

that T cells spontaneously trend to express IFNg rather under neutral/ Th0 conditions (at least compared to the release of other T helper subset defining cytokines like IL17a, IL4, IL-22...), I wonder what the effect of the "basal" Batf expression originating from TCR activation is in this regard. As it is now under Th17 conditions, IL-6/ Stat3 driven effects - thereby enhancing and prolonging Batf expression but potentially not only this axis alone- are not allowing to decipher the contribution of TCR activated Batf for suppressing a Th1 like signature. So, is the signature and protein imprint (Fig. 1C, top row) also visible under neutral conditions in the absence of Batf compared to Batf-sufficient T cells?
2. Over the last 10 years or so, there is a broad set of literature on various molecular contributions of Batf as a critical TF regulating the development, homing and functionality of FoxP3+ T cells. Hence, it appears unlikely that the observed regulation of Foxp3 (in the Schraml et al. data set) is meaningful especially given the not convincing flow cytometric analyses shown in Fig 1C, bottom row, with a merely moderate increase observed in the absence of Batf. It is not about doubting the results, it is about doubting the relevance for Batf deficiency related T helper cell lineage specification. Along these lines it is an overstatement to say that in the oral C. rodentium infection model the absence of Batf may also account for tempered Th17/Th22 responses not through the well-established mechanism underlying Th17/22 differentiation but also through unleashed FoxP3+ T cell formation that suppress Th17/22 cells further. There is no evidence for that. Moreover, the evidence provided for the conclusion that Batf deficient T cells in this model are forced into FoxP3+ Treg formation compared to Batf competent controls (Fig. 1O) is weak given the provided FACS blots. Are these functionally Tregs? Are the number of Tregs absolutely enhanced in the colon of Batf deficient mice in this model to name a few additions that need to be made so that the data are more convincing and fitting the conclusions and interpretations. In my mind, the infectious colitis model serves the purpose to make the point that also in vivo Batf-deficient T cells display a Th1-like effector T cell phenotype. Regardless, this qualitatively (and quantitatively?) gain of Th1 like cells is unable to control infection while lack of infection control is most likely due to hampered Th17/22 development and functionality but may also be in part result from a defective humoral and innate response. The germline model of Batf deficiency does not allow to differentiate between these possibilities. Overall, the data allow correlative statements of the T cell and colitis phenotype but both aspects cannot be brought into a functional relationship. In the light of comments also under 1. and the provided data in the remaining figures, I suggest that the manuscript/ study focusses on the Th1 like T cell phenotypes in the absence of Batf.
3. Data shown in Figure 2E-F and 3A-B and resp. Fig EV3 are not convincingly showing that Batf deficient T cells display a meaningful regulation of CD25 expression (increase) and IL-2 (increase). Also here, stating that regulating IL-2/IL-2R levels may represent a critical, indirect mechanism underlying Batf-dependent Th17 lineage specification, is in my regard not covered by the provided data and too tendentious. For example, de-regulated JAK mediated activation and/ or defective inactivation (i.e.dephosphorylation) of activated Stat5 (via SOCS) may be an alternative/ additional cause underlying prolonged and increased Stat5 dependent regulation of gene expression-mediated effects in the absence of Batf. Moreover, the Schraml study (Fig 1B) and Kurachi et al, NI 2014, reported that type I interferon response is markedly enhanced in T cells deficient for Batf and Batf is involved in its regulation resp.. Also, besides IFNg also the IFNg receptor gene contains Batf binding sites und its expression seems to be induced in the absence of Batf. Hence, e.g. enhanced IFNg/IFNgR signaling might reinforce a Th1 like phenotype. Overall, the molecular basis for the Th1 like phenotype and enhanced Stat5 occupancy remains to me not fully elucidated, more controls and alternative scenarios alongside the Stat5 signaling pathway may be tested to provide further support for the conclusions of the authors.
4. In Fig 3D showing CHIP seq. data from Th17 cells it is -intrinsic to the chosen experimental approach- problematic that IL-2 and IL-6 mediated Stat5 and Stat3 recruitment resp. are tested in Th17 cells at d5 that per definition represent diametrically opposed T cells from a biological point of view given the abrogated ability of Batf deficient T cells to become a Th17 cell. Hence, to clearly show that Batf directly controls Stat5 activation (or Batf deficiency mediates Stat5 activation, as the title of the figure 3 may be revised to), an earlier time point or a different experimental setting/ approach (e.g. TCR activated T cells only) should be taken to convincingly rule out a bias due to the comparison of Th17+ vs Th17- T cells. Moreover, to me in Fig 3 D, upper row, left panel (IFNg) the interpretation that Stat3 binding was observed in a restricted manner, while Stat5 binding increased throughout the loci is also an overstatement given the displayed result chart showing that there is visibly as well a substantial increase of Stat3 binding to the IFNg locus.
5. Interestingly, the Ets1/Runx1/ Stat5 complex is shown to be involved in driving a Th1 like T cell phenotype found in "failed" Th17 cells due to the absence of Batf. The study provides data stating that in regard to expression levels of Ets1/Runx1, general chromatin accessibility of respective Th1 like loci characteristic for Batf deficient T cells (d5, Th17 differentiation) and also binding of respective TF to Th1 like gene loci are not dramatically regulated and hence not likely to underlie the observed Th1 like gene expression profile in the absence of Batf. Hence enhanced Stat5 signaling has been put forward to represent the key mechanism- both pharmaceutical inhibition of Stat5 and antibody-mediated blockade of CD25 signaling have been employed to provide evidence for the functional role of Stat5. Finally the functional contribution of Runx1 and Ets1 have been underscored through siRNA experiments. These studies are together convincing and elegant but it remains unaddressed in what way the defined complex of TF - Runx1, Ets1 and Stat5 - cooperate to induce the observed outcome. Is a physical interaction of Stat5 and Ets1 required or only of Runx1 and Ets1. Given the fact that the authors have cStat5ko T cells at hand and have used the tool of retroviral reconstitution experiments it was interesting to assess with the help of loss of function variants (Stat5) to start to gain insight into the molecular requirements underlying the three-molecule interaction. As it is now, conclusions in regard to that are purely speculative.
6. Fig 2 E &F are mentioned/ discussed in the results section before data from Fig. 2C/D are reported. The figure order need to be adjusted to account for that.

Referee #3:

This paper is well written, the study outlined is easy to follow, and the data presented is clear. The work complements prior studies showing that IL-2 and STAT5 can modulate TH17 plasticity and commitment (and Th1/Treg differentiation). It also adds to work by this group already establishing a link between BATF and Ets1 and Th differentiation. While the work appears rigorous in most areas, there are a few controls that would seem important to include to further increase the rigor (i.e. use of STAT5-deficient cells, unstimulated controls, BATF-Chip in BATF-KO and STAT5-ChIP in STAT5CKO cells etc..). I list some of these in the concerns below for reference. My major concern is that I do not get a sense from the work shown how these in vitro studies translate into in vivo biology. Much of the basis for this BATF/IL-2R-driven mechanism has already been flushed out and thus, this paper is relying on cementing this mechanism. Unfortunately, a lot of what is provided is still descriptive (albeit supportive) of a potential mechanism. Certainly, there is good evidence that BATF and STAT5 are working together to enhance gene expression, but whether this is a key pathway in driving the plasticity between Th17 and Th1/Treg development (certainly IL-2 is already known to do this) is not clear beyond what is already known. The differences attributed to the effects are often 2-fold (look at RT-PCR and culture supernatant data). While 2-fold can have biologic significance, in a confined, in vitro system like this, it likely suggests that the mechanism tweaks rather than drives a phenotype. Thus, more effort to establish this pathway/mechanism in vivo seems important or mutation of the stat5 binding sites to show this enhancesome is critical would be sufficient. A better assessment of these pathways and their effect on Th17 plasticity in vivo or a more clear assessment of the requirement for the enhancesome binding at these sites would significantly increase my enthusiasm for the manuscript. In addition, more single cell assessment (ICS for example) would aid in the understanding of what the results from bulk culture systems, CHIP-seq, ATAC-seq, RT-PCR, and cytokine levels in superantants mean across all cells in the culture (particularly important as only 30% of cells are confirmed as Th17 (via IL-17 after PMA/ionomycin restim) after Th17 culture. I have provided some of the more critical concerns below, that if addressed, would immediately increase the impact of these findings.

Major Concerns:

1) The gene expression data in Figure 1 is done using bulk RNAseq or RT-PCR of bulk cultures. Bulk analyses preclude an ability to assess the heterogeneity within a population. Given the cytokine profiles provided in Figure 1C, only 30% of WT cells show IL-17 even after PMA/ionomycin. This would indicate that the majority of T cells are not committed Th17 cells. Thus, while bulk data is informative, the low degree of polarization in the Th17 cultures (even for WT cells) makes interpretations somewhat limited. This is particularly true as no unstimulated control culture was provided as reference. Is there a baseline effect of having no BATF? Is BATF expressed similarly in the IL-17+ vs IL-17- cells. If so, what does this indicate with regard to your system and hypothesis? This concern holds for the siRNA studies as well. In the absence of scRNAseq data, unstimulated controls of WT and BATFKO should be used to assess what the baseline transcriptomes are like in WT and KO. Similarly, a comparison of IL-17+ vs IL-17- would be interesting given degree of heterogeneity among the cultures. This could be done with IL-17A reporters (Fig. 1F example).
2) Figure 1O shows CD25 (IL2R) staining. It is not obvious to me if there is a difference in expression across timepoints between BATFKO and WT. This would seem to contradict the mechanism being raised in the rest of the paper? Is this a difference between in vivo and in vitro findings due to intracellular CD25 vs surface expression?
3) Given the importance of IL-2R in the conclusions of the paper, Figure 2C-D is important to place into context. Given that only 30% of the cultures are Th17 cells, what constitutes the non-bimodal shift in CD25 (see bimodal staining for p-STAT data as comparison in Figure 3A and lack of difference in CD25 and p-STAT5 in sup. Figure 3A, 3C))? Is this difference in receptor expression significant biologically when only observed in late stage culture? Can you show differences in STAT5-phosphorylation when titrating IL-2 into the culture?
4) Again, given the importance given to changes in IL-2R in BATFKO vs WT Th17 differentiation (or repression of Th1/Treg) for the rationale of this mechanism, Figure 2E-H should be complemented with single cell protein values (flow data). This would provide more more context into the biology than transcript expression alone.
5) Given hypothesis that BATF binding influences STAT5 binding which is important during Th17 differentiation (or Th1/Treg suppression), Figure 3C-D would benefit from seeing what the baseline peaks look like without addition of IL-2 and IL-6. This would strengthen the hypothesis. Similarly, figures do not show CHIP-seq for BATF in the BATFKO cells. Antibodies used in CHIP-seq rarely produce no background. This should be shown to give the reader context. This is especially true given noted background in Sup. Fig 6B (ChIP %input).
6) Methods seem to suggest that no IL-2 is provided during Th17 culture conditions. Given the hypothesis that IL-2R signaling is critical for Th17 vs Th1/Treg plasticity in the absence of BATF, how does this fit into the mechanism? Where does the IL-2R signal come from? Would one expect to see differences in STAT5 binding in BATFKO and WT if no IL-2 signaling is taking place over the course of the culture? What would happen if IL-2 was added during the Th17 cultures? Is the IL-2 being provided from cells in the culture? Does this effect go away if anti-IL2 is added throughout Th17 culture conditions?
7) In figure 6 and 7, it is difficult to know how the RT-PCR and cytokine levels in bulk culture supernatant results reflect biology using stat5 inhibitor in vitro. As the authors suggest, STAT5 is known to promote Th1/Treg and inhibit Th17. This data is certainly important and supporting, but it remains unclear how these changes impact Th17 development in vivo? Similarly, what does this mean across all cells in the culture (is this a few cells affected or all (ICS staining would be helpful)?
8) Seems many of these experiments (especially Figure 6 and 7) would benefit using STAT5-deficient cells as controls (see mouse in methods). The inhibitor studies are interesting, but Sup. Fig 6A is not sufficient to show effectiveness. This should be done with and without IL-2 stimulation. This should give a very nice + control for real p-STAT5 staining.

9) Transfection efficiency should be provided for all relevant experiments.

Minor Concerns:

1) Figure 1A does not have a marker for BATFKO and WT. Presumably the grey is WT as BATF was observed, but this should be stated in the figure or the legend.
2) In the absence of BATF, there are some studies that suggest that BATF2 and BATF3 can compensate. Your data would suggest that this does not happen. This should be commented on in the manuscript.
3) Figure 1C: Does BATF expression differentially correlate with the IL-17+ cells relative to the IL-17 negative? If not, please comment.
4) Figure 1E: The data in Figure 1C is striking but Figure 1E is difficult to place in that context. IFNg shows a <2x effect in the supernatants yet a 20-fold effect between KO and WT in the ICS staining. What percentage of cells were transfected? Does this account for the small differences as compared to the pervious data? Transfection efficiency should be provided also for Figure 2E etc...
5) It is unclear why ATAC-seq data was provided at 2 days and CHIP-seq data was provided at 5 days. Seems paired days is more appropriate and informative.
6) In Figure 2B it is difficult to understand what to focus on. Nothing is highlighted in the figure to direct the reader to differences in the ATACseq and histone modifications at the IL-2R locus. Please indicate by boxes or arrows what areas are important and significantly different (please provide how significance was achieved).
7) Figure 7 says BATF regulates the STAT5 in Th17 cells. The text states that BATF (shown in figure 1K by RT-PCR) is decreased without IL-6 and TGF-beta. For rigor, this statement needs to be backed up by protein analysis on a single cell level as this figure is trying to make the point that BATF is driving the STAT5 phenotype.

Response to Reviewers

We thank each of the referees for taking time to consider our study and for their thoughtful assessment and recommendations for its improvement. We have made considerable effort to address each of the concerns, performing a number of additional studies and providing new data where feasible. We hope you share our enthusiasm that the study has been substantially impproved in revision. Here we address each reviewer's comments point by point, restating each of the comments in *italics* and responding in plain text. Thanks again for reconsidering our work.

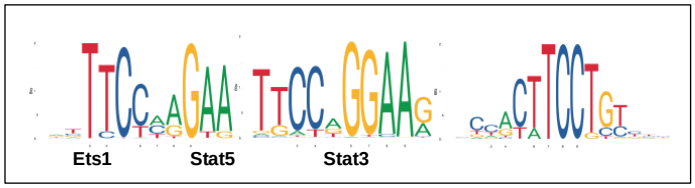
Referee #1:

In this study, Pham et al examine the mechanisms by which Batf regulates CD4 helper cell differentiation and Th17 cell stabilization. The authors show that Batf deficiency results in upregulation of IFNg- and Foxp3-transcriptomic modules and reduction in expression of genes participating in the Th17 program (e.g. Il23r, Rorc, Maf, Il17a, Il17f and IL21). These findings were confirmed using siRNA and ectopic expression of Batf. In vivo, Batf deficiency was associated with impaired host defense against C. rodentium and aberrant helper T cell differentiation. The authors next employ Chip-seq for Batf and histone modifications (in WT and knockout cells) and ATACseq in in vitro differentiated Th17 cells. The authors show that Batf binds the Il2ra locus and its absence is associated with loss of repressive marks with increased expression in vitro and in vivo. This is associated with increased STAT5 phosphorylation and genomic binding, along with increased accessibility and deposition of H3K27Ac surrounding Th1 and Treg loci. STAT5 Chipseq in Batf deficient cells shows enrichment of Runx and Ets motifs and pharmacologic inhibition of Stat5 increased binding of Batf and STAT3 to the Il17 locus. In the context of their previous demonstration of instability of Th17 cells over time, the authors show that in the absence of IL-6 and TGFb, Batf expression declines over time with the enhancement of IL-2/Stat5 signals and recruitment of Ets1 and Runx1 to Th1 and Treg loci.

This manuscript tackles an important issue and provides interesting new mechanistic insights. In general, the manuscript is well-written and thoughtful and provides comprehensive and convincing data for the proposed mechanism. However, the authors should consider the following points:

1. *It is unclear why the authors define STAT motifs as a half site and not a full palindrome. Do the authors believe that STATs are predominantly functioning as monomers partnered with Ets or Runx rather than as STAT dimers?*

First, we thank the reviewer for their positive assessment of the original manuscript and their appreciation of the new mechanistic insights generated and for the suggestions for improvement and are grateful for the thoughtful recommendation for improvement. We also thank the reviewer for posing this interesting question. We have included here the full palindromic Stat3 and Stat5 consensus sequences to more clearly demonstrate that the Ets1 core sequence mirrors that of the Stat3/5 half site. The sequence logos in Figure 4 represent *composite* sites generated from motif analyses of Ets1 with Stat3 and/or Stat5 co-bound regions in WT (F), Batf KO (G) or Stat5 KO (H) Th17 cells and therefore display the Stat3/5 half sites in combination with the Ets1 site. While unphosphorylated Stat3 can translocate into the nucleus and bind to DNA as a monomer (Timofeeva et al., JBC 2012, Nkansah, E Febs Letters 2013) and Stat5 monomers can also cycle to the nucleus (Zeng et al., JI 2002), cytokine signaling-induced phosphorylation leads to dimerization in the cytoplasm followed by nuclear translocation, forming the majority of Stat3 and Stat5 in the nucleus. The anti-Stat3 and anti-Stat5 (detects both Stat5a and Stat5b) antibodies used in these ChIP experiments were not specific for phosphorylated proteins so we cannot rule out the



possibility that Stat3/5 monomers partner with Ets1/Runx1 at selected sites throughout the genome. However, given the exposure to IL-6 and IL-2 directly prior to the ChIP procedure, nuclear Stat3 and Stat5 likely bind with Ets1 in dimeric form.

2. *If feasible, the in vivo significance of the work might be enhanced by including ATACseq and transcriptomic data using cells from Citrobacter infected Batf-deficient mice.*

We agree that it might be beneficial to probe the effects of Batf on chromatin reorganization during *Citrobacter* infection, although the rationale for including these data was to demonstrate that the phenotype of the infected Batf-deficient animals is consistent with our in vitro studies. Due to the substantial time and cost to generate sufficient age-/sex-matched WT and Batf KO mice with littermate controls—in large part due to a microbiota shift in our colony that has complicated *Citrobacter* studies—and the anticipation of limited additional mechanistic insights without performing companion ChIP-seq studies that have not been feasible due to the inability to obtain sufficient cell numbers for quality data, we have respectfully opted to forego these experiments.

3. *The gating on IL-22 producers is confusing; it is unclear whether all cells produce small amounts of IL-22 or the major peak represents non-producers; clarification is necessary. Based on ATACseq and Chip-seq data is the Il22 locus closed?*

Thanks for raising this point; we agree. To clarify the gating on the IL-22-producing cells, we have now included intracellular staining for IL-22 in naïve CD4$^+$ cells as a negative control (Expanded View Figure 1I). Specifically, the flow cytometry profiles show that on day 10 post infection, a notable fraction of the CD4 T cells in the colon from wild type animals express IL-22 whereas IL-22 expression is undetectable in naïve CD4 T cells and in colonic CD4 T cells from Batf KO animals.

While we do not have ATAC-seq or ChIP-seq data from Batf-deficient cells post *Cr* infection (see comment, above), our published studies (Schraml et al., Nature 2009) and data not shown demonstrate that Batf binds to the *Il22* promoter in Th17 cells and, in absence of Batf, IL-22/*Il22* expression is diminished. Given the role of Batf as a pioneer factor for this gene, it is likely that accessibility at the *Il22* locus is similarly diminished in CD4 Batf KO T cells during *Cr* infection.

4. *The Stat5 inhibitor used is an antipsychotic drug identified in a repurposing screen. It is unclear why this approach was used rather than STAT5 cKO mice or alternatively STAT5 siRNA; either would be useful to verify the results with pimozide.*

This is a valid point. Our studies demonstrate that in the absence of Batf, Stat5 recruitment to Th1- and Treg-specifying loci is enhanced in Batf KO cells cultured under Th17 conditions. Ets1 and Runx1 motifs were found to be enriched in the Stat5-bound regions and we subsequently showed that Ets1 and Runx1 are similarly recruited to these same loci. We wished to determine whether Stat5 directed the assembly of an Ets1/Runx1 containing enhanceosome that promoted Th1- and Treg-like profiles in the absence of Batf. We chose to pharmacologically inhibit Stat5 rather than breeding Batf KO animals to Stat5$^{fl/fl}$ *Cd4*-Cre mice to generate “double knockout” mice in the interest of the substantial time and cost that would be incurred to develop this line. Note, however, that we have established that chemical inhibition of Stat5 effectively diminished pStat5 (see revised Expanded View Figure 5A), reduced Stat5 genomic binding (Figure 6C) and impacted *IL17a*/IL-17a and *Ifng*/ IFN-γ expression (Figure 6A,B) similarly to that observed in Stat5cKO Th17 cells (Expanded View Figure 5D,E). With regard to using siRNA to diminish Stat5 expression, we routinely achieve gene knock-down efficiencies similar to that observed using the Stat5 inhibitor (40-50%). For many of the experiments, the Stat5 inhibitor was

favored over siRNA knock down to be able to target Stat5 at the initial stage polarization instead of transfecting siRNA on d3.

5. *The scale depicted in Figure 6 minimizes differences in STAT3 binding; the authors should be more circumspect in their conclusions.*

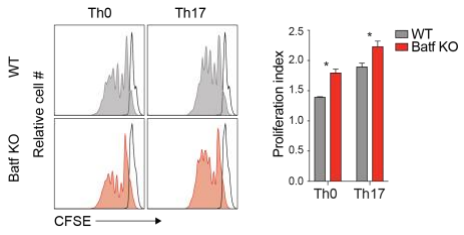
The scale of the y-axis was initially kept similar to that of Stat3 binding at the *Il17a* locus to emphasize that the binding of Stat3 was significantly lower at Th1- and Treg-related gene loci compared to the *Il17a/f* locus (see Figure 6C vs. Expanded View Figure 6C). However, we agree that this could be confusing and have changed the scale of the y-axis in Figure 6C regarding the Stat3 binding per the reviewer's suggestion. This resulted in no significant difference between the groups.

Minor points/suggestions:
6. Global Batf deletion negatively impacts ILCs; the authors should consider whether phenotype seen in Figure 1 may also be result of impaired ILC3 function.

This is an excellent point. We agree with the reviewer that it is possible that a diminished presence of ILC early in infection (day 5) contributes to increased inflammation due to reduced IL-22 production. We have now addressed this in the results section.

7. *Given the impact of Batf on IL-2 signaling, might it be informative to show CFSE along with cytokine production?*

Based on our findings of increased IL-2 production in BatfKO cells (Figure 2J), we agree that cellular proliferation could be impacted. To test this, CFSE labeled WT and Batf-deficient naïve CD4 T cells were cultured under Th0 or Th17 conditions and assessed for proliferation by CFSE dilution on d3 (right). In accord with enhanced IL-2 expression, BatfKO cells showed a modest increase in proliferation. However, it should be noted that we have not observed an appreciable difference in cell number or viability in BatfKO versus WT Th17 cultures. If the reviewer thinks these data should be included in the manuscript, we will be happy to incorporate them.



Batf-deficient CD4 T cells exhibit a modest increase in cell proliferation. Naïve WT and Batf KO CD4^{+}CD62^{hi} T cells were labeled with CFSE and cultured under neutral (Th0) or Th17 polarizing conditions. Cell proliferation was determined after three days by flow cytometry.

8. *Anti-IFNg antibody is included in Th17 cultures but given that Batf deficiency potently enhances IFNg production, the question arises whether dysregulation of helper cell programs may in part be due to IFNg. How confident are the authors in terms of the efficacy of blockade? Controls reinforcing this point could be useful.*

Another excellent point. We have now added a new figure (Expanded View Figure 1D) demonstrating that IFN-γ expression in both WT and BatfKO Th0 is completely abolished when anti-IFN-γ is included in the culture.

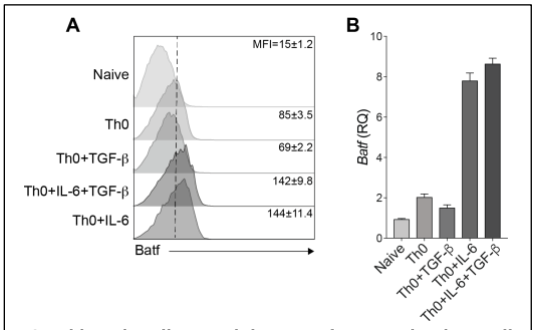
9. *In figure 1, it would likely be of interest to identify/label the most highly induced genes.*

Thank you. Per this suggestion, we have now added gene names for the most-induced genes in WT and Batf KO cells in Figure 1A.

10. *What is the relative importance of IL-6 vs. IL-23 on Batf expression; does IL-6 alone induce Batf*

and what if any, is the role of TGFb? Is the effect of TGFb principally related to inhibition of IFNg rather than promoting Batf expression?

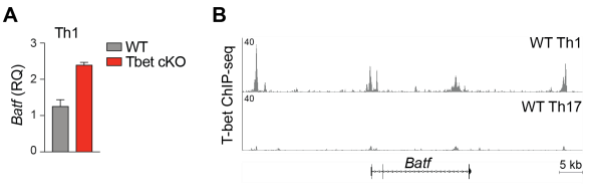
Excellent quesiton. In a previous study (Pham et al., Cell Reports 2019) we showed that IL-6 signaling potently upregulates Batf. To confirm this finding and to investigate the effects of TGF-β on Batf expression, we cultured naïve CD4 T cells under neutral conditions with or without the addition of IL-6 and/or TGF-β (right). As we have described, IL-6 induced robust Batf expression whereas TGF-β alone or in combination with IL-6 did not promote Batf expression and may have in fact, modestly reduced its expression. In another published study (Harbour, et. al., Sci Immunol 2020), we showed that ongoing IL-6 signaling is essential to maintain the Th17 phenotype by sustaining Rorγt expression, which in turn induces IL-23R expression. Both IL-6 and IL-23 signal through pStat3 and can support Batf expression (Li et. al., Cell Mol Immunol. 2018; Pham et al., Cell Reports 2019). However, while the amplification of TCR-induced Batf expression by IL-6 is foundational to the Th17 phenotype, *Il23r* expression is dependent on IL-6 induced Rorγt and thereby acts later in development.



Cytokine signaling modulates Batf expression in T cell Naïve WT CD4$^+$ T cells were cultured under neutral conditions or with the indicated cytokines. On day 5, cells were stained intracellularly to measure Batf expression represented by histograms with quantified mean fluorescence intensity (MFI) (**A**) or were assayed for *Batf* transcript expression by qRT-PCR (**B**)

11. *Tbx21 is not altered by Batf deficiency; however, is the converse operative? Using public datasets does T-bet directly regulate Batf or is this largely mediated by Ifng?*

This is another very interesting point; thank you. In new experiments (right), we analyzed *Tbx21*-deficient Th1 cells and found that *Batf* expression is increased in the absence of T-bet. We further demonstrated that T-bet binds to the *Batf* locus in Th1 cells. Together these results suggest that T-bet might actively repress *Batf* in Th1 cells, although additional experiments are needed to validate these findings.



Cytokine signaling modulates Batf expression in T cells. WT or *Tbx21* cKO naïve CD4 T cells were cultured under Th1 or Th7 conditions. On day 5, Th1 cells were collected and analyzed for *Batf* transcript expression by qRT-PCR (**A**). ChiP-seq was performed on d5 Th1 or Th17 cells and Tbet recruitment to the *Batf* locus was assessed (**B**).

12. *The data provided in Figure 2 would be strengthened by quantifying and displaying overall differential accessibility.*

In published studies now referenced in the text (Pham et al., Cell Reports 2019), we extensively analyzed and quantified the effects of Batf deficiency on chromatin accessibility in Th17 cells. Briefly, we demonstrated that greater than 30% of accessible chromatin is lost in the absence of Batf and that these Batf-dependent regions were enriched at transcriptional start sites, particularly those of genes previously identified to be regulated by Batf.

13. *Figure 7B: no cumulative data are provided relating to pStat5 levels. It is notable that the relatively high expression of pStat5 in Th17(R3) cells does not correlate with low level of Il2ra (Figure 7A). Some explanation is warranted.*

In reference to the request for cumulative data, we have revised this figure, replacing *Il2ra* transcript data with CD25 expression assessed by flow cytometry with MFI quantification (Figure 7A), and thank the reviewer for the suggestion. In new figures 7A & B, pStat5 expression Th17(R3) is significantly less than in TCR(R3). However as pointed out by the reviewer, the reduction in pStat5 does not entirely

correlate with the more substantial diminution of CD25 (*Il2ra* in the original submission). We have found in other studies (Dean *et al.*, in preparation) that modulation of the IL-2Rβ and γ_c chains and potentially SOCS factors contribute considerably to pStat5 after the primary round of polarization and suspect that the same may be operative here, alhtough this would require further study. We direct the referee to our response to similar concerns by referee 2 below (item #3).

14. *Figure 1O: cumulative data of percent IFNg+, IL-17a+, and CD25+ Foxp3+ cells in WT and Baft KO are not provided.*

The requested data can now be found in Expanded View Figure 1H.

15. *The titles for Figures 5 and 6 are identical. Better articulation of the take-home message would help. Also, no subheadings are used in the results section; subheadings would improve readability. More paragraph breaks would also be helpful, especially when the manuscript is in its published format.*

We thank the reviewer for noting this oversight! Figure 6 now has a new title that more accurately reflects the data presented.

Referee #2:

The study "Batf stabilizes the Th17 developmental program through impairment of Stat5-dependent recruitment of Ets-Runx1 complexes" by Pham addresses so far unstudied aspects alongside differentiation of T helper cells into Th17 cells previously reported to be pioneered by the AP1 transcription factor Batf together with IRF4. This study was motivated by the observation that T helper cells deficient for Batf display a gene expression signature suggestive of a Th1 /Treg phenotype. Mechanistically the authors identify that T cells deficient for Batf express enhanced levels of IL-2ra/CD25 and argue that this condition may underlie the cells' increased permissiveness for IL-2/Stat5 signaling, especially fueling Th1 and Treg related gene loci. Mechanistically, enhanced Stat5 binding of Th1/Treg differentiation related loci in the absence of Batf was accompanied by increased Ets1 and Runx1 containing occupancies and Th1 /Treg related gene expression profiles. Blocking CD25 and Stat5 signaling directly reduced Th1/Treg prone gene signature of Batf deficient T cells cultured under Th17 conditions.

This study is giving interesting and so far not reported insight into the multi-faceted functionality of Batf driven T helper lineage specification. Addressing this important area is highly relevant but following concerns and suggestions may be addressed or followed up respectively to strengthen the significance and specificity of the results presented in that manuscript.

We thank this reviewer for recognizing the novelty and importance of our study, and we appreciate the thoughtful recommendations for its improvement. We are particularly excited that the study is much improved by inclusion of new experiments that have directly addressed cooperativity of Ets1-Runx1-Stat5 in response to this referee and referee #3 (see revised Figure 6), which we believe are novel and important.

1*. Re-analyzing microarray data from Ken Murphy's group (Fig. 1A/B) showing for the first time the critical role for Batf during Th17 differentiation the authors made the observation that Batf deficient T cells polarized under Th17 conditions display a Th1 like signature. Given the pioneering function of Batf in the context of gene regulation in T cells and the well- known observation that T cells spontaneously trend to express IFNg rather under neutral/ Th0 conditions (at least compared to the release of other T helper subset defining cytokines like IL17a, IL4, IL-22...), I wonder what the effect of the "basal" Batf*

expression originating from TCR activation is in this regard. As it is now under Th17 conditions, IL-6/ Stat3 driven effects - thereby enhancing and prolonging Batf expression but potentially not only this axis alone- are not allowing to decipher the contribution of TCR activated Batf for suppressing a Th1 like signature. So, is the signature and protein imprint (Fig. 1C, top row) also visible under neutral conditions in the absence of Batf compared to Batf-sufficient T cells?

We agree that TCR-driven Batf expression deserves attention, particularly with regard to Th1 development given the increased IFN-γ expression we find in Batf-deficient Th17 polarized cells. In new data (Expanded View Figure 1D), we show that Batf KO CD4 T cells grown in neutral conditions express higher levels of IFN-γ than WT cells suggesting that Batf may directly suppress IFN-γ in CD4 T cells perhaps contributing along with modulation of the IL-2 expression/signaling pathway to stabilize the Th17 phenotype.

2*. Over the last 10 years or so, there is a broad set of literature on various molecular contributions of Batf as a critical TF regulating the development, homing and functionality of FoxP3+ T cells. Hence, it appears unlikely that the observed regulation of Foxp3 (in the Schraml et al. data set) is meaningful especially given the not convincing flow cytometric analyses shown in Fig 1C, bottom row, with a merely moderate increase observed in the absence of Batf. It is not about doubting the results, it is about doubting the relevance for Batf deficiency related T helper cell lineage specification. Along these lines it is an overstatement to say that in the oral C. rodentium infection model the absence of Batf may also account for tempered Th17/Th22 responses not through the well-established mechanism underlying Th17/22 differentiation but also through unleashed FoxP3+ T cell formation that suppress Th17/22 cells further. There is no evidence for that. Moreover, the evidence provided for the conclusion that Batf deficient T cells in this model are forced into FoxP3+ Treg formation compared to Batf competent controls (Fig. 1O) is weak given the provided FACS blots. Are these functionally Tregs? Are the number of Tregs absolutely enhanced in the colon of Batf deficient mice in this model to name a few additions that need to be made so that the data are more convincing and fitting the conclusions and interpretations. In my mind, the infectious colitis model serves the purpose to make the point that also in vivo Batf-deficient T cells display a Th1-like effector T cell phenotype. Regardless, this qualitatively (and quantitatively?) gain of Th1 like cells is unable to control infection while lack of infection control is most likely due to hampered Th17/22 development and functionality but may also be in part result from a defective humoral and innate response. The germline model of Batf deficiency does not allow to differentiate between these possibilities. Overall, the data allow correlative statements of the T cell and colitis phenotype but both aspects cannot be brought into a functional relationship. In the light of comments also under 1. and the provided data in the remaining figures, I suggest that the manuscript/ study focusses on the Th1 like T cell phenotypes in the absence of Batf.*

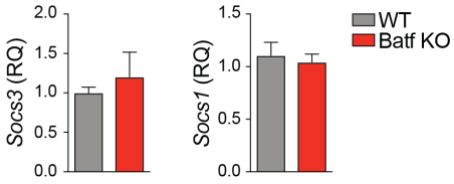
We appreciate the reviewer's insightful comments regarding increased Treg development in the *Cr* model. We agree that IFN-γ-producing cells are the most prominent CD4 T cell population that develops in the gut of *Cr*-infected BatfKO animals and have now emphasized this more clearly in the revised text. However, we do find that the absolute number of Foxp3-expressing cells in the colon is significantly increased in Batf KO cells compared to WT cells in the *Cr* model (Expanded View Figure 1G) though it is true that functional consequence on Th17/Th22 development has not been directly assessed. Thus, we have modified the text to more accurately reflect the data presented. We have also improved the Treg flow cytometry profiles that we hope the reviewer will find more convincing.

3*. Data shown in Figure 2E-F and 3A-B and resp. Fig EV3 are not convincingly showing that Batf deficient T cells display a meaningful regulation of CD25 expression (increase) and IL-2 (increase). Also here, stating that regulating IL-2/IL-2R levels may represent a critical, indirect mechanism underlying Batf-dependent Th17 lineage specification, is in my regard not covered by the provided data*

and too tendentious. For example, de-regulated JAK mediated activation and/ or defective inactivation (i.e.dephosphorylation) of activated Stat5 (via SOCS) may be an alternative/ additional cause underlying prolonged and increased Stat5 dependent regulation of gene expression-mediated effects in the absence of Batf. Moreover, the Schraml study (Fig 1B) and Kurachi et al, NI 2014, reported that type I interferon response is markedly enhanced in T cells deficient for Batf and Batf is involved in its regulation resp.. Also, besides IFNg also the IFNg receptor gene contains Batf binding sites und its expression seems to be induced in the absence of Batf. Hence, e.g. enhanced IFNg/IFNgR signaling might reinforce a Th1 like phenotype. Overall, the molecular basis for the Th1 like phenotype and enhanced Stat5 occupancy remains to me not fully elucidated, more controls and alternative scenarios alongside the Stat5 signaling pathway may be tested to provide further support for the conclusions of the authors.

We acknowledge that the increases in IL-2 and IL-2R expression in BatfKO Th17 cells presented in our original submission were modest, though we respectfully disagree that this enhancement is not meaningful. In other studies of IL-2R components in eTreg development (Dean *et al.*, in preparation) we find significant alteration in the trajectory of eTreg cell development contingent on similarly "modest" shifts in receptor expression. In any case, revised Figure 2 now includes new data (E, F, H,K-L) that more convincingly demonstrate that Batf functions to repress *Il2* expression early in Th17 differentiation and IL-2R later in development. In Figure 2E, siRNA knock down of Batf on d3 post Th17 polarization resulted in increased CD25 expression whereas over-expression of Batf diminished CD25 (Figure 2F). Together with Batf recruitment to the *Il2ra* and *Il2rb* loci (Figure 2B, Expanded View 3D) and decreased H3K27me3 marks and increased H3K27ac recruitment in BatfKO cells, collectively these data support a direct role for Batf in the suppression of the IL-2R in Th17 cells. Also, in new Figure 2K, the *Il2* locus was found to be more accessible in Batf-deficient cells two days following Th17 polarization, correlating with increased IL-2 reporter expression on d1 and 2 (Figure 2J). Th17 cells cultured for an additional two rounds without exogenous cytokine where Batf expression wanes (Figure 1I, L) also produce more IL-2 than cells continuously grown with Th17-polarizing cytokines (Figure 2L) or that over-express Batf (Figure 2M). These data indicate that Batf curbs CD25 expression first by limiting IL-2 production that reduces autocrine induction of the IL-2R and then later, by directly repressing *Il2ra*.

We also think it important to note here that our data are consistent with previous studies demonstrating increased CD25/*Il2ra* in Treg (Wheaton, 2020) and CD8 T cells (Kurachi 2014) in the absence of Batf/Ap1 factors. As noted, we had overlooked the Socs-dependent dephosphorylation of Stat5 as a contributing mechanism to the Batf-deficient phenotype and thank the reviewer for this helpful suggestion. We have now assessed Socs1 and Socs3 expression in BatfKO Th17 cells (right) and find little difference in expression compared to WT cells, suggesting these proteins do not contribute to the increased pStat5 observed in our system. As discussed in our response to query 1 and 2, and based on our data that demonstrates that Batf is recruited to the *Ifng* locus concurrently with repressive histone marks (Figure 2B), we suggest that Batf may directly repress *Ifng* expression thereby contributing Th17 lineage stabilization. We have stated this more explicitly in the revised text.



Similar Socs1 and Socs3 expression in the absence of Batf in T cells. Naïve WT and Batf KO CD4^{+}CD62^{hi}T cells cultured under Th17 polarizing conditions for 5 days were analyzed for Socs1 and Socs3 expression by qRT-PCR.

4*. In Fig 3D showing CHIP seq. data from Th17 cells it is -intrinsic to the chosen experimental approach-problematic that IL-2 and IL-6 mediated Stat5 and Stat3 recruitment resp. are tested in Th17 cells at d5 that per definition represent diametrically opposed T cells from a biological point of view given the abrogated ability of Batf deficient T cells to become a Th17 cell. Hence, to clearly show that Batf directly controls Stat5 activation (or Batf deficiency mediates Stat5 activation, as the title of the figure 3 may be revised to), an earlier time point or a different experimental setting/ approach (e.g. TCR activated T*

cells only) should be taken to convincingly rule out a bias due to the comparison of Th17+ vs Th17- T cells. Moreover, to me in Fig 3 D, upper row, left panel (IFNg) the interpretation that Stat3 binding was observed in a restricted manner, while Stat5 binding increased throughout the loci is also an overstatement given the displayed result chart showing that there is visibly as well a substantial increase of Stat3 binding to the IFNg locus.

We appreciate the reviewer's concern regarding the restimulation of our d5 BatfKO and WT Th17 cultures. The purpose of reactivating Stat5 and Stat3 with IL-2 and IL6, respectively, prior to ChIP was to amplify the signal to noise ratio in the ChIP-seq data. As discussed in response to query 3, the increase in CD25 expression in BatfKO Th17 cells occurs late in development and therefore d5 was selected for these experiments. In new studies, we performed Stat5 ChIP comparing d5 WT and Batf KO Th0 cells. Similar to Th17 cells, Stat5 binding at the *Ifng* locus was significantly increased in Batf KO cells (Expanded View Figure 3E) supporting our findings that Batf represses Stat5 activation indirectly through modulation of the IL-2 receptor.

Stat3 and Stat5 have been shown to have opposing effects on genome occupancy at the *Il17a/f* loci (Yang et al., 2011). However, despite increased Stat5 binding (Figure 3C), we did not observe reduced Stat3 recruitment at Th1 and Treg-related genomic regions. Thus, our data implied that increased Stat5 binding in Batf KO cells occurs through a Stat3-independent mechanism. We too had observed *increased* Stat3 binding at areas in the *Ifng* and *Il18r1* loci along with enhanced Stat5 recruitment throughout and was stated so in the text. Importantly, the level of Stat3 binding at Th1-like loci in Batf KO cells was substantially lower than at the *Il17a/f* locus in WT cells (Figure 6C vs. Expanded View Figure 5C; and Figure 3D) though it is unclear at this time what the implications of Stat3 recruitment to these loci are. We respectfully suggest that studies to address this are beyond the scope of the current report.

5*. Interestingly, the Ets1/Runx1/ Stat5 complex is shown to be involved in driving a Th1-like T cell phenotype found in "failed" Th17 cells due to the absence of Batf. The study provides data stating that in regard to expression levels of Ets1/Runx1, general chromatin accessibility of respective Th1 like loci characteristic for Batf deficient T cells (d5, Th17 differentiation) and also binding of respective TF to Th1 like gene loci are not dramatically regulated and hence not likely to underlie the observed Th1 like gene expression profile in the absence of Batf. Hence enhanced Stat5 signaling has been put forward to represent the key mechanism- both pharmaceutical inhibition of Stat5 and antibody-mediated blockade of CD25 signaling have been employed to provide evidence for the functional role of Stat5. Finally the functional contribution of Runx1 and Ets1 have been underscored through siRNA experiments. These studies are together convincing and elegant but it remains unaddressed in what way the defined complex of TF - Runx1, Ets1 and Stat5 - cooperate to induce the observed outcome. Is a physical interaction of Stat5 and Ets1 required or only of Runx1 and Ets1. Given the fact that the authors have cStat5ko T cells at hand and have used the tool of retroviral reconstitution experiments it was interesting to assess with the help of loss of function variants (Stat5) to start to gain insight into the molecular requirements underlying the three-molecule interaction. As it is now, conclusions in regard to that are purely speculative.*

We appreciate the reviewer's enthusiastic comments regarding the Batf-modulated Ets1-Runx1-Stat5 enhancer complex that we have identified herein and agree that this is a major component of the advance of this study. Thank you! To extend and further support this finding, we have now performed a suite of experiments using DNA Affinity Precipitation Assay (DAPA) to interrogate the association of the three transcription factors at selected genomic sites (new Figure 6D, E). These studies identified clear cooperativity between Ets1, Runx1, and Stat5 at relevant target sequences. Accordingly, mutation of the Ets1 or Stat5 binding sites at these elements significantly impaired binding of the other proteins.

Additionally, we have included a new figure from studies using Stat5cKO cells to assess and validate the requirement of Stat5 for Ets1 recruitment to Th1- and Treg-like loci (Expanded View Figure5G). Consistent with our Stat5 inhibitor experiments, Ets1 binding was decreased in Stat5cKO cells cultured under Th17 conditions. We also performed Stat5 ChIP-qPCR in Batf KO cells with diminished Ets1 and Runx1 expression (siRNA knockdown) and showed reduced Stat5 binding compared to control-treated Batf KO cells in new Figure 6F. Together these new data reinforce our conclusion that Batf stabilizes the Th17 phenotype by modulating the IL-2 receptor and its downstream signalis, thereby preventing the assembly of an Ets1-Runx1-Stat5 enhancer complex that promotes alternative developmental programming.

6*. Fig 2 E &F are mentioned/discussed in the results section before data from Fig. 2C/D are reported. The figure order needs to be adjusted to account for that.*

We thank the reviewer for noting the order of the figures. The text has been appropriately amended.

Referee #3:

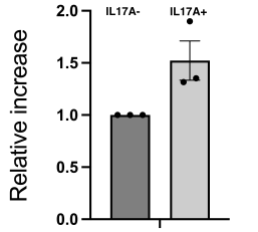
This paper is well written, the study outlined is easy to follow, and the data presented is clear. The work complements prior studies showing that IL-2 and STAT5 can modulate TH17 plasticity and commitment (and Th1/Treg differentiation). It also adds to work by this group already establishing a link between BATF and Ets1 and Th differentiation. While the work appears rigorous in most areas, there are a few controls that would seem important to include to further increase the rigor (i.e. use of STAT5-deficient cells, unstimulated controls, BATF-Chip in BATF-KO and STAT5-ChIP in STAT5CKO cells etc..). I list some of these in the concerns below for reference. My major concern is that I do not get a sense from the work shown how these in vitro studies translate into in vivo biology. Much of the basis for this BATF/IL-2R-driven mechanism has already been flushed out and thus, this paper is relying on cementing this mechanism. Unfortunately, a lot of what is provided is still descriptive (albeit supportive) of a potential mechanism. Certainly, there is good evidence that BATF and STAT5 are working together to enhance gene expression, but whether this is a key pathway in driving the plasticity between Th17 and Th1/Treg development (certainly IL-2 is already known to do this) is not clear beyond what is already known. The differences attributed to the effects are often 2-fold (look at RT-PCR and culture supernatant data). While 2-fold can have biologic significance, in a confined, in vitro system like this, it likely suggests that the mechanism tweaks rather than drives a phenotype. Thus, more effort to establish this pathway/mechanism in vivo seems important or mutation of the stat5 binding sites to show this enhancesome is critical would be sufficient. A better assessment of these pathways and their effect on Th17 plasticity in vivo or a more clear assessment of the requirement for the enhancesome binding at these sites would significantly increase my enthusiasm for the manuscript. In addition, more single cell assessment (ICS for example) would aid in the understanding of what the results from bulk culture systems, CHIP-seq, ATAC-seq, RT-PCR, and cytokine levels in superantants mean across all cells in the culture (particularly important as only 30% of cells are confirmed as Th17 (via IL-17 after PMA/ionomycin restim) after Th17 culture. I have provided some of the more critical concerns below, that if addressed, would immediately increase the impact of these findings.

Thank you for the very thoughtful comments. While we respectfully suggest that major aspects of our study are no more "descriptive" than much of the literature that addresses Th17 development, we also hope this referee shares are excited about new data included in revision and hope the importance and novelty of the work is evident by these additional data, particularly the new experiments in Figure 6 that address similar issues raised by Referee 2 (see response to item 5, above) which now directly demonstrate cooperativity of Ets1, Runx1 and Stat5 at target sequences and show that mutation of Stat5 binding sites greatly diminished Ets1 and Runx1 recruitment.

Major Concerns:

1*. The gene expression data in Figure 1 is done using bulk RNAseq or RT-PCR of bulk cultures. Bulk analyses preclude an ability to assess the heterogeneity within a population. Given the cytokine profiles provided in Figure 1C, only 30% of WT cells show IL-17 even after PMA/ionomycin. This would indicate that the majority of T cells are not committed Th17 cells. Thus, while bulk data is informative, the low degree of polarization in the Th17 cultures (even for WT cells) makes interpretations somewhat limited. This is particularly true as no unstimulated control culture was provided as reference. Is there a baseline effect of having no BATF? Is BATF expressed similarly in the IL-17+ vs IL-17- cells. If so, what does this indicate with regard to your system and hypothesis? This concern holds for the siRNA studies as well. In the absence of scRNAseq data, unstimulated controls of WT and BATFKO should be used to assess what the baseline transcriptomes are like in WT and KO. Similarly, a comparison of IL-17+ vs IL-17- would be interesting given degree of heterogeneity among the cultures. This could be done with IL-17A reporters (Fig. 1F example).*

It has been established and is generally accepted in the field that *in vitro* Th17 cell culture conditions typically yield ~20-50% IL-17a$^+$ cells after acute PMA/ionomycin restimulation, whereas expression of IL-17f is considerably higher (>75%), as originally reported by us (Lee at al., *Immunity* 2009). Thus, we respectfully disagree that the majority of the cells in the culture are not committed to the Th17 phenotype. Moreover, in another of our studies (Mukasa *et al*, *Immunity* 2010) we demonstrated that the genomic landscape of bulk Th17 cells was similar to that of cells that had been isolated based on *Il17f* expression. Finally, sorted IL-17+ and IL-17- T cells used in a recent scRNA-seq study demonstrated minimal differences in the transcriptomes of the two populations and only a slight increase in *Batf* expression in the IL17+ population. We have validated these findings by qRT-PCR (right). Thus, we propose that the heterogeneous expression of IL-17a in *in vitro* generated Th17 cells does not accurately reflect Th17 commitment and does not substantially impact our data interpretation. As suggested, we have now included RNA-seq analysis of d5 resting WT and Batf KO Th17 cells (Expanded View Figure 1A). Note that the gene expression profiles were similar between unstimulated and stimulated cells (Figure 1A), showing enhanced Th1- and Treg-like gene expression profiles in Batf KO cells compared to WT cells.



Similar *Batf* expression in IL-17+ VS IL-17- Th17 cells. Naïve CD4+ T cells isolated from *Il17a*^{GFP} mice were cultured under Th17 conditions for 3 days, sorted on GFP expression and analyzed for *Batf* expression by qRT-PCR.

2*. Figure 1O shows CD25 (IL2R) staining. It is not obvious to me if there is a difference in expression across timepoints between BATFKO and WT. This would seem to contradict the mechanism being raised in the rest of the paper? Is this a difference between in vivo and in vitro findings due to intracellular CD25 vs surface expression?*

Batf functions as an essential pioneering factor in the development of Th17 cells, hence the paucity of Th17 cells in BatfKO animals. Without Batf, *Cr*-infected animals defaulted to a primarily Th1-like response but also have increased numbers of Foxp3+ regulatory cells, represented in Figure 1O (now Expanded View 1G). The CD25+Foxp3- cells in the panels from BatfKO animals represent the expanding, highly activated Th1 population (Figure 1P). The mechanism we are proposing centers on Batf repressing IL-2R in late stage Th17 cells, functioning as a phenotype stabilizer. Since Th17 cells do not develop in *Cr*-infected Batf KO animals and we have not proposed that this mechanism is shared in Th1 or Treg cells, we respectfully disagree that these data are contradictory. With regard to Tregs, Batf expression is low (Figure 2G) and in studies we have not included here, Batf deficiency did not substantially impact *Il2ra* express in Treg. Also, we stress that CD25 expression was assessed by cell surface staining in all of our experiments.

3*. Given the importance of IL-2R in the conclusions of the paper, Figure 2C-D is important to place into context. Given that only 30% of the cultures are Th17 cells, what constitutes the non-bimodal shift in CD25 (see bimodal staining for p-STAT data as comparison in Figure 3A and lack of difference in CD25 and p-STAT5 in sup. Figure 3A, 3C))? Is this difference in receptor expression significant biologically when only observed in late stage culture? Can you show differences in STAT5-phosphorylation when titrating IL-2 into the culture?*

As noted above (response to item #1), the fraction of Th17 cells in these cultures far exceeds 30% as IL-17a is only expressed by a subset of developing Th17 cells. Because TCR signaling induces the expression of IL-2 and CD25 on CD4 T cells that act in a feed-forward loop, IL-2-Stat5 signaling enhances the expression of CD25 on the cell surface. Our studies demonstrate that CD25 expression was expressed at a very high level as early as 4 h after TCR stimulation, while pStat5 was nearly undetectable (Expanded View Figure 3A, C) suggesting an early disconnect with CD25 expression, IL-2 availability and pStat5 likely account for the non-bimodal shift in CD25 expression.

While IL-2 contributes to T cell proliferation and expansion, it also inhibits Th17 cell development, thus tight regulation of the IL-2-Stat5 axis is essential for the stability of this phenotype. In WT Th17 cells cultured with strong TCR stimulation, IL-2 production peaked by day 2 as did CD25 and pStat5 activation (Figure 2J, Expanded View Figure 3A-C). As activated T cells acquired a Th17 phenotype in these experiments, the level of CD25 and pStat5 rapidly declined while Batf expression was sustained. Our studies further indicated that Batf recruitment to the *Il2ra* locus accumulated over time and was associated with enhanced H3K27me3 deposition. Given the inherent plasticity of late stage Th17 cells, we suggest that it is plausible for mechanisms that stabilize phenotypic fate arise later in development.

As for the last query, regarding whether we can show differences in STAT5 phosphorylation with titrating IL-2 into the culture, the short answer is “no” but this is not because our model is incorrect. While we have hypothesized that BatfKO cells would phosphorylate Stat5 at a lower IL-2 dose than WT cells due to enhanced expression of CD25, these studies have been confounded by the inability to completely block the endogenous expression of IL-2. Th17 cells express substantial amounts of IL-2 (DiToro *et al*, Science 2018) and even at the highest doses of IL-2 blocking antibody (S4B6), pStat5 was still detected. We then sought to generate Th17 cells from IL-2-deficient naïve CD4 T cells to eliminate the contribution of endogenous IL-2. However, interestingly, Th17 cell development was completely blunted in the absence of IL-2 and was not adequately rescued by exogenous addition of IL-2, suggesting a developmental aberration in T cells from these mice that could not be rescued by exogenous IL-2. At present, we do not know the basis for this disconnect and it deserves further study. Suffice ot sya that we agree that this is an important line of investigation, however with current reagents and tools, we are unable to resolve this.

4. *Again, given the importance given to changes in IL-2R in BATFKO vs. WT Th17 differentiation (or repression of Th1/Treg) for the rationale of this mechanism, Figure 2E-H should be complemented with single cell protein values (flow data). This would provide more more context into the biology than transcript expression alone.*

We thank the reviewer for the suggestion and have replaced RT-PCR data with flow cytometry data to assess CD25 protein expression (Figure 2E, F, and H).

5*. Given hypothesis that BATF binding influences STAT5 binding which is important during Th17 differentiation (or Th1/Treg suppression), Figure 3C-D would benefit from seeing what the baseline peaks look like without addition of IL-2 and IL-6. This would strengthen the hypothesis. Similarly, figures do not show CHIP-seq for BATF in the BATFKO cells. Antibodies used in CHIP-seq rarely produce no background. This should be shown to give the reader context. This is especially true given noted background in Sup. Fig 6B (ChIP %input).*

This is an excellent point. As discussed in the response to Referee #2's question 4, stimulating WT and BatfKO cells with IL-2 and IL-6 prior to performing the ChIP was to generate a more robust Stat5 and Stat3 signal. We agree that it would be informative to identify the "baseline" Stat3 and Stat5 peaks but in several experiments, we have been unable to immunoprecipitate enough DNA to make representative libraries, consistent with minimal Stat3 or Stat5 binding at baseline.

When our lab first developed the Batf antibody (Schraml et al., Nature 2009) we performed Batf ChiP-seq using BatfKO cells. The libraries that were generated were not diverse, with areas of the genome being over amplified, indicating that the antibody does have some background, however, the specificity of our Batf polyclonal antibody in ChIP experiments has been repeatedly confirmed (Schraml et al., Nature 2009, Ise et al Nature Immunology 2011, and Pham et al., Cell Reports 2019).

6. *Methods seem to suggest that no IL-2 is provided during Th17 culture conditions. Given the hypothesis that IL-2R signaling is critical for Th17 vs Th1/Treg plasticity in the absence of BATF, how does this fit into the mechanism? Where does the IL-2R signal come from? Would one expect to see differences in STAT5 binding in BATFKO and WT if no IL-2 signaling is taking place over the course of the culture? What would happen if IL-2 was added during the Th17 cultures? Is the IL-2 being provided from cells in the culture? Does this effect go away if anti-IL2 is added throughout Th17 culture conditions?*

We apologize for the oversight in neglecting to include this crucial information in our original submission. hIL-2 was added at 10 U/ml to the culture on day 3 during Th17 cell differentiation to promote cell survival in the in vitro culture assay. The amended text now reflects this correction. Moreover, as discussed in response to question 3, Th17 cells express substantial amounts IL-2 within two days of the initiation of the culture. Batf-deficient cells produce even greater amounts of IL-2 and therefore the initial amplification of CD25 expression in BatfKO cells is due to the increased IL-2 being produced by the cells in the culture.

7. *In figure 6 and 7, it is difficult to know how the RT-PCR and cytokine levels in bulk culture supernatant results reflect biology using stat5 inhibitor in vitro. As the authors suggest, STAT5 is known to promote Th1/Treg and inhibit Th17. This data is certainly important and supporting, but it remains unclear how these changes impact Th17 development in vivo? Similarly, what does this mean across all cells in the culture (is this a few cells affected or all (ICS staining would be helpful)?*

Another excellent point. We have now compared IL-17a and IFN-γ expression in Stat5 inhibitor treated to Stat5-deficient in T cells (Stat5cKO; Figure 6A, B with Expanded View Figure D and Expanded View B with Expanded View E) and have found them to be remarkably similar. ICS of Th17 cells either cultured with the Stat5i or derived from Stat5 deficient T cells (Expanded View Figures B, E) demonstrated an approximately 30% increase in IL-17a expression. Published studies by Laurence et al (Immunity 2007) showed that CD4 T cells isolated from $Stat5^{fl/fl}$ X CD4-Cre mice exhibited an activated phenotype and were predisposed to develop into the Th17 phenotype. Serum from these mice also had higher levels of IL-17 protein than littermate controls. These studies clearly demonstrate that Stat5 impacts Th17 development *in vivo*. We suggest that during an inflammatory response, Th17 cells are maintained by continued IL-6 signaling and induction of Batf that in turn decreases the sensitivity to IL-2 signaling. As the local cytokine environment evolves, diminishing IL-6 gives way to alternative fates depending on the nearby factors. Our data implicate reduced Batf triggering enhanced CD25 as an important step in allowing this transition.

8. *Seems many of these experiments (especially Figure 6 and 7) would benefit using STAT5-deficient cells as controls (see mouse in methods). The inhibitor studies are interesting, but Sup. Fig 6A is not sufficient to show effectiveness. This should be done with and without IL-2 stimulation. This should give a very nice + control for real p-STAT5 staining.*

We thank the reviewer for this suggestion. Regarding the control for pStat5 staining, we have added naïve cells as the negative control for Figure 7B and Expanded View Figure 6A and as discussed in the response to query 7, we have validated similar effects of the Stat5 inhibitor and endogenous Stat5 deletion. The primary reason for choosing to use the Stat5 inhibitor was because we wished to demonstrate that the phenotypic changes observed in Batf deficient cells were, in part due to increased pStat5. Because of financial constraints we are unable to develop *Batf*$^{-/-}$.*Stat5*$^{fl/fl}$.CD4-Cre double deficient animals. Based on the similar effect of the Stat5 inhibitor we felt confident that the inhibitor was a suitable substitute for many of our studies.

9. *Transfection efficiency should be provided for all relevant experiments.*

Our transfection efficiency of approximately 30%-50% is consistent with established Amaxa (Lonza) mouse T cell nucleofection. The siRNA experiments are validated by assessing the targeted gene expression by qRT-PCR (Batf, ~40% Figure 1E; Ets1 and Runx1, ~50% Expanded View Figure 6B).

Minor Concerns:
1. *Figure 1A does not have a marker for BATFKO and WT. Presumably the grey is WT as BATF was observed, but this should be stated in the figure or the legend.*

We thank the reviewer for noting the omission! We have now labeled Figure 1A appropriately.

2. *In the absence of BATF, there are some studies that suggest that BATF2 and BATF3 can compensate. Your data would suggest that this does not happen. This should be commented on in the manuscript.*

This is an excellent point. Our RNA-seq data indicated that *Batf2* expression is negligible in Th17 cells, while *Batf3* expression was significantly lower in Batf KO cells compared to WT cells suggesting that Batf2 and Batf3 cannot compensate for Batf in our studies. A comment to this effect is now included in the main text.

3*. Figure 1C: Does BATF expression differentially correlate with the IL-17+ cells relative to the IL-17 negative? If not, please comment.*

As discussed in the response to question 1, *Batf* expression is modestly increased in IL-17+ cells.

4. *Figure 1E: The data in Figure 1C is striking but Figure 1E is difficult to place in that context. IFNg shows a <2x effect in the supernatants yet a 20-fold effect between KO and WT in the ICS staining. What percentage of cells were transfected? Does this account for the small differences as compared to the pervious data? Transfection efficiency should be provided also for Figure 2E etc...*

Good point; thanks reviewer pointing out the difference. siRNA targeting Batf in Th17 cells resulted in ~40% reduction in Batf expression (revised Figure 1E), likely contributing to the less robust expression of IFN-γ compared to Batf-deficient cells.

5. *It is unclear why ATAC-seq data was provided at 2 days and CHIP-seq data was provided at 5 days. Seems paired days is more appropriate and informative.*

This is a valid point. However, in previous studies we found that Batf to remodels chromatin at Th17-specifying loci within 48 hours of the initiation of Th17 polarization and that the genomic landscape remains relatively stable thereafter (Pham et al., *Cell Reports* 2019; Mukasa *et al*, *Immunity* 2010). Our current studies demonstrate that while the accessibility of Th17 gene loci is substantially diminished, Th1- and Treg-like loci are minimally affected by Batf deficiency. In trying to understand the propensity for Th17 cells to deviate from their original program, we focused on changes that occur later in development, which better coincided with our finding that Batf-dependent regulation of the IL-2 receptor signaling complex and its Stat5 output modulated downstream transcriptional effectors during this later time window, hence our interrogation of these factors by ChIP-seq studies in this time window. We agree that an argument can be made for time matching the ATAC- and ChIP -seq studies. However, we feel strongly that the rationale for the two time points is valid and we opted to avoid the high cost of performing another replicate set of ATAC-seq and ChIP-seq at the coordinated time points.

6. *In Figure 2B, it is difficult to understand what to focus on. Nothing is highlighted in the figure to direct the reader to differences in the ATACseq and histone modifications at the IL-2R locus. Please indicate by boxes or arrows what areas are important and significantly different (please provide how significance was achieved).*

As suggested, we have added shading to Figure 2B to indicate significant differential peaks.

7. *Figure 7 says BATF regulates the STAT5 in Th17 cells. The text states that BATF (shown in figure 1K by RT-PCR) is decreased without IL-6 and TGF-beta. For rigor, this statement needs to be backed up by protein analysis on a single cell level as this figure is trying to make the point that BATF is driving the STAT5 phenotype.*

As suggested, we have replaced *Batf* transcript expression data with intracellular Batf protein expression in Figures 1I and 1L. We have moved the previous panels, 1H and 1K to Expanded View Figure 1.

References

DiToro et al., *Science* 2018 Sep; 361(6407):eaao2933
Harbour, et. al., *Sci Immunol* 2020 Jul; 5(49):eaaw2262
Ise et al *Nat Immunol* 2011 Jun; 12(6):536-43
Kurachi et al., *Nat Immunol* 2014 Apr; 15(4):373-83
Laurence et al., *Immunity* 2007 Mar; 26(3):371-81
Lee at al., *Immunity* 2009 Jan; 30(1):92-107
Li et al., *Front Immunol* 2022 Apr; 13:882721
Liu et al., *Sci Immunol* 2020 Dec; 5(54): eaaz8154
Mukasa et al., *Immunity* 2010 May; 32(5):616-27
Nkansah, E *Febs Letters* 2013 Apr; 587(7): 833-839
Owen and Farrar. *F1000Res* 2017 Jan;6:32
Pham et al., *Cell Reports* 2019 Oct; 29(5):1203-1220.e7
Schraml et al., *Nature* 2009 Jul;460(7253):405-9
Timofeeva et al., *JBC* 2012 Apr; 287(17):14192-14200
Wang et al., *J Exp Med* 2013 Mar; 210(3):475-89
Wheaton and Ciofani, *Front Immunol* 2020 Mar; 11:444
Zeng et al., *J Immunol* 2002 May; 168 (9) 4567-4575

Dear Casey,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the original referees #2&3. As you can see from the comments below, the referees appreciate the introduced changes and support publication here.

I am therefore very pleased to accept the MS for publication here. Before sending you the formal acceptance letter there are just a few editorial points that need to be resolved:

- The email to Daniel J Silberger bounced please fix

- you can only have 5 keywords

- Please relabel Accession codes as data availability

- COI should be re-labeled as Disclosure and competing interests statement

- Please remove the Authors Contributions from the manuscript. The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')

- Please fix format of reference list to EJ style

- We don't allow data not shown (pgs 11 and 15). Please re-phrase

- Please make sure that the author checklist is filled out.

- The figures need to be uploaded as single high-resolution files.

- Please fix call out to Figure 2F - currently says Fig. F. Also check callouts to Figs EV1E,F, EV3E, EV5D-G

- "Methods" needs correcting to Materials and Methods.

- The Reference section should come directly before the Figure Legends.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

- Fig EV2D => we don't encourage statistics when n=2. Can you show both data points?

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Can you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- I also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels).

That should be all! When you submit the revised version, please also include a point-by-point response to the editorial points.

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #2:

In fact the manuscript significantly improved upon the revision process. The major conclusions are covered by the data although in regard to the IL-2/IL-2R axis, as implied by the authors themselves the impact of Batf had been implicated before.Regardless the provided data represents an important puzzle piece to the understanding of Batf driven Th17 biology and should be accepted for publication.

Referee #3:

While there are still some concerns with respect to overall novelty and the in vivo validation of the in vitro findings to substantiate the proposed mechanism, the authors for the most part were very responsive to prior critiques. The new experiments add to the substantial data already provided, and I believe increase the potential impact of the findings. This is certainly of interest to the general EMBO audience. As such, I feel the current manuscript has sufficiently addressed my concerns and should be considered for publication.

All editorial and formatting issues were resolved by the authors.

2nd Revision - Editorial Decision **19th Jan 2023**

Dear Robin and Casey,

Thank you for submitting your revised manuscript. I have now had a chance to take a careful look at everything and all looks good.

I am therefore very pleased to accept the MS for publication here.

Congratulations on a nice study!

PS let me know if you want to submit a different synopsis image.

Best

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

EMBO Press Author Checklist

Corresponding Author Name: Robin Hatton and Casey Weaver
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-109803R

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 $\chi 2$ 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Methods
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure Legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure Legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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	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	