nature research

Peer Review Information

Journal: Nature Immunology
Manuscript Title: BATF and IRF4 cooperate to counter exhaustion in tumour-infiltrating CAR T cells
Corresponding author name(s): Anjana Rao, Patrick G Hogan

Editorial Notes:

Redactions – unpublished data	Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Decisions:

Decision Letter, initial version:

Subject: Decision on Nature Immunology submission NI-A30443-T
Message: 18th Sep 2020

Dear Anjana & Patrick,

Thank you for supplying a point-by-point response to the referees' comments on your manuscript entitled "BATF and IRF4 cooperate to counter exhaustion in tumour-infiltrating CAR T cells". As noted in my previous message, while they find your work of considerable potential interest, they have raised quite substantial concerns that must be addressed. In light of these comments, we cannot accept the current manuscript for publication, but would be very interested in considering a revised version that addresses these concerns as outlined in your response.

We hope you will find the referees' comments useful as you decide how to proceed. If you wish to submit a substantially revised manuscript, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions.

Specifically, as noted in your response:
(1) ChIP-seq and RNA-seq data and analysis for CAR T cells overexpressing BATF-HKE
(2) Bioinformatic analysis of the genes co-targeted by BATF and IRF4, and the correlation between transcription factor binding and changes in gene expression
(3) Bioinformatic analysis of the correlation between BATF binding and changes in chromatin accessibility
(4) Replication of the experiments on BATF-overexpressing CAR T cells in B16 tumors

(5) Data showing the similar effects of BATF overexpression in an additional tumor model
(6) A test of whether BATF overexpression has similar effects in human CAR T cells
(7) A more thorough explanation of the evidence pointing to BATF-IRF cooperation.

When you revise your manuscript, please take into account all reviewer and editor comments, please highlight all changes in the manuscript text file in Microsoft Word format.

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Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further.

Thank you for the opportunity to review your work.

Kind regards,

Laurie

Laurie A. Dempsey, Ph.D.
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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The manuscript by the Rao and Hogan labs is focused on the transcriptional programming of tumor infiltrating CAR-T cells, specifically on mechanisms that can be used to overcome their hypo-sensitization (exhaustion) thereby enhancing their efficacy from a therapeutic standpoint. The problem is of considerable translational interest and also raises fundamental issues that pertain to negative feedback control of effector T cell responses. The manuscript generates clear insight into the translational aspect but does not sufficiently explore the fundamental issues.
The authors begin by following up on earlier work which showed that CD8 CAR-T cells which are deleted for Tox or Nr4a family transcription factors, exhibit increased effector function and enhanced anti-tumor responses. Candidate transcription factors whose expression is upregulated by deletion of Tox or Nr4a genes are tested individually, using a

gain-of-function approach to determine which can increase the effector functions of CAR-T CD8 cells in mouse models. This results in the main finding that BATF overexpression in CD8 CAR-T cells promotes their survival and expansion in the tumor microenvironment, increases production of effector cytokines, diminishes expression of inhibitory receptors and leads to the generation of long-lived memory T cells that can control recurrence of tumors. These findings are extensively documented and some are nicely extended to BATF over-expressing OT-I cells.
The remaining manuscript turns to the molecular mechanism(s) by which BATF over expression and importantly its sustained expression in tumor infiltrating CD8 T cells causes these phenotypic changes in the effector capacity of the cells and overcomes their propensity to undergo exhaustion. The most insightful experiment in this regard is the use of a BATF mutant protein which has previously been shown to be defective it its interaction with the transcription factor IRF4. This BATF mutant fails to enhance the expansion and effector capacity of the engineered CAR-T cells leading to the conclusion that BATF-IRF4 cooperate as molecular complexes to counteract CD8 T cell exhaustion. From a mechanistic standpoint the following issues remain unresolved:

1. Which genes are directly co-targeted and co-regulated by BATF and IRF4 and likely account for the increased proliferative capacity of the CAR-T cells in the tumor microenvironment versus their enhanced effector functions and also the ability to generate memory. The RNA-seq and ChIP-seq experiments attempt to address these issues but do not analyze genes impacted by manipulating IRF4 levels. Furthermore, such target genes that are of strongest biological interest in the three fore-mentioned categories need to be highlighted.
2. Additional experiments with the BATF mutant protein would enhance mechanistic insight. This would involve performing ChIP-seq and RNA-seq experiments with such engineered TILs (earlier timepoints after adoptive transfer) to determine if the mutant BATF protein fails to bind to many or all sites that IRF4 is bound to or perhaps the mutant protein causes diminished IRF4 binding. This could then be connected to the alterations in gene expression. This experiment is particularly important as the authors suggest that endogenous BATF is sufficient to enable IRF4 binding to regions that contain AICE motifs recognized by BATF-IRF4 complexes.

From a biological and therefore more fundamental standpoints the following issues remain to be addressed:

1. Is it increased expression of BATF or its sustained expression in CAR-T cells that results in their enhanced proliferation and effector functions? Does altering the levels of ectopically expressed BATF affect the various phenotypic properties?
2. Can induction of BATF in exhausted CAR-T cells revive their proliferative and effector capacities? Is the exhausted state of a TIL reversed by BATF from an epigenetic and transcriptional standpoint?
3. What is the molecular mechanism by which BATF is repressed in exhausted CD8 T cells such that continued TCR signaling resulting in the activation of NFAT but not AP-1 fails to induce BATF? The direct dependency for its repression by Tox and/or Nr4a family members is not explored.

Reviewer #2:
Remarks to the Author:

Much attention is now being focussed on the function of basic leucine zipper (bZip) transcription factors of the AP-1 family in regulating CD8+ T cell responses to chronic stimuli. Indeed the authors' earlier work has shown that while the TF NFAT plays a critical role in driving both T cell activation and exhaustion, the relative extent to which it drives either programme is affected by the levels to which NFAT is accompanied by its dimerisation partner, the AP-1 TF c-Jun (Martinez et al., Immunity 2015). As a consequence, it is now known that overexpression of c-Jun augments CD8+ T cell function upon adoptive immunotherapy, by limiting exhaustion (Lynn et al., Nature 2019).

This manuscript by Seo et al. extends this earlier work, examining the ability of various AP-1 factors when overexpressed to augment anti-tumour immunity upon adoptive immunotherapy. They find that a different bZip TF, BATF, when overexpressed in CD8+ T cells augments their ability to mediate anti-tumour immune responses upon adoptive immunotherapy using two mouse models - a anti-CD19 CAR transduction / B16-hCD19 solid tumour model and the OT-1 TCR-tg / B16-Ova models and is superior to overexpression of Jun TFs, leading to striking tumour rejection.

The significance of the work should be considered in the context of prior work in this area: The relationship between BATF and CD8+ T cell function has been examined in several prior studies, each with somewhat different conclusions. Originally, it was proposed that BATF drives T cell exhaustion (Quigley Nat Med 2010). However, it has also been shown using gene knockout studies in mice that BATF is required for acute expansion of CD8+ T cells early following T cell stimulation (Kurachi et al., Nat Immunol 2014). BATF is known to participate in IRF4/JunD/BATF heterotrimers at DNA whose differential affinity for distinct enhancer sequences determines the extent to which weak TCR signals drive gene expression (Iwata et al., Nat Immunol 2017). Finally, it has been shown that REGNASE-1 deletion in CD8+ T cells in adoptive immunotherapy dramatically improves anti-tumour efficacy in a manner partially dependent upon increased BATF expression, since combined loss of BATF and REGNASE-1 reverse the phenotype resulting from REGNASE-1 deficiency alone (Wei et al., Nature 2019).

Regardless of these prior findings, however, the value of the work is that it defines a novel way to augment the anti-tumour function of CD8+ T cells and in the models used, this appears to be superior to the effect of c-Jun overexpression which has been the subject of quite intense interest. Thus, the manuscript is of interest to the field. However, the manuscript is rather correlative in nature, and a general model of how BATF promotes CD8+ T cell function in the context of adoptive immunotherapy does not clearly emerge from the data presented. In addition, while it is proposed that reduction in the exhausted state of CD8 T cells is a mechanism by which BATF overexpression augments T cell survival and immunotherapy, this has not been experimentally demonstrated.

Major comments:
BATF overexpressing cells have superior survival and effector function upon adoptive immunotherapy, and the authors correlate these observations with decreased expression of exhaustion markers, including PD-1, and TOX. It is therefore proposed that BATF functions by limiting exhaustion. The authors would need to justify these comments experimentally - ie is the phenotype (increased survival) reversed upon restoration of PD-1 expression, or phenocopied (in terms of T cell survival and anti-tumour efficacy) upon genetic ablation of PD-1 or TOX? These are important experiments especially given the authors observe decreased TOX levels in BATF-overexpressing cells. Loss of TOX expression has previously been shown to result in decreased CD8+ T cell survival (Scott et

al., Nature 2019). How do the authors reconcile these observations?

The authors see loss of memory-associated molecules including TCF-1, CD62L and CD127 and express increased expression of KLRG-1, associated with terminal differentiation, at early timepoints - observations that are somewhat difficult to reconcile with the superior survival of BATF-overexpressing cells. At late timepoints, increased frequencies of memory cells are observed. How does BATF promote increased survival of T cell responses? Can the authors propose a model, justified by new experimental data, for how BATF overexpression promotes CD8+ T cell survival and effector function? Is it primarily through driving a larger acute expansion phase of the response? Is this function independent of phenotype, and is it primarily through increased proliferation or decreased apoptosis? Whilst they use Ki67 staining for showing that there is an increase in CAR T cell proliferation, the median increase is of 1.5 fold (Fig 1J). Is such an increase enough to allow for the huge increase in survival?

3. The reporter system to test the activity of bZip TFs of interest in driving gene expression by NFAT:AP-1 sites (Fig S1A-F) utilised a retroviral vector with tandem NFAT:AP-1 sites driving expression of both Thy1.1 and the bZip TF whose function was being tested - resulting in a positive feedback loop as the authors state. This feedback loop precludes straightforward interpretation of the experimental results. A simpler reporter system should be developed where a constitutive promoter drives expression of the bZip TF of interest allowing the activity of different TFs to be tested in a fair comparison absent of such positive feedback artefact.

4. Fig. 1. Is the expression (in terms of MFI or percentage expression) of the transduced CAR affected by the co-transduction with the various bZip factors? Data could be shown

5. Fig. 3B. It is known that inoculation of killed B16 cells generates immunity to further B16 administration, so it is not clear from the data presented that the surviving T cell response drives de novo rejection of the B16-Ova secondary tumour. A good control would be to implant either B16-Ova or B16 control lines into animals that rejected the initial B16-Ova tumours to demonstrate that it is the Ova-specific response that caused rejection of the tumours.

6. Fig. S4D. The accessibility of BATF-bound sites at the TOX locus was decreased in the absence of BATF. Is this locus specific? What is the general function of BATF in regulating chromatin accessibility at its binding sites? Ie can bioinformatic analysis of ATAC-Seq data at BATF sites be performed to examine whether BATF-bound sites generally increase or decrease in occupancy when BATF is overexpressed?

7. When BATF is overexpressed, is the binding of another TF reduced, whose function is to subvert T cell effector function, promote exhaustion marker expression and limit survival? Ie is a a competition model with another bZip TF most relevant - in which case, can ChIP-Seq experiments be performed to examine what factors bind less when BATF is overexpressed? Or is NFAT binding alone in the absence of BATF? Can this be experimentally demonstrated? Can ATAC-footprinting be informative here?

8. Fig. 5. Whilst there is a clear difference between the outcome of BATF and BATF-HKE overexpression, further experiments to confirm the hypothesis disruption of BATF and IRF4 binding is "absolutely required" would be necessary, particularly since this is one of

the main findings and thrust of the paper. Is it possible that the HKE mutation, under overexpression conditions, could disrupt some other function of BATF other than the IRF4 interaction? It could be of interest to test whether cells carrying IRF4 mutatations (instead of BATF mutants) unable to form the BATF-IRF4 association would lead to the same outcome as using BATF-HKE mutants.

As a control, the authors should test whether the HKE mutations in BATF result in its diminished expression or DNA binding in T cells - these are important controls in interpreting the profound loss of function when BATF-HKE is overexpressed. Only in the case that these are unaffected can it be stated that the interaction with IRF4 is necessary for the observed function.

It may be interesting to study differences in the transcriptome upon overexpression of BATF and BATF-HKE, as this could provide evidence of how the HKE mutants differ from BATF-transduced cells. Is there a decrease in the transcription levels of IRF4-bound genes in HKE mutants compared to BATF CAR T cells?

9. BATF WT and HKE overexpressing cells both diminished PD-1 expression but had divergent effects on survival - what does this mean? Does it mean that the function of BATF WT in promoting survival has nothing to do with its effect on PD-1 expression? Effects of PD-1 on survival could be discussed in the context of Scott et al. (Nature, 2019) and Odorizzi et al. (JEM, 2015), where loss of exhaustion marker expression is associated with diminished - not enhanced - survival.

10. ChIP analyses show that overexpression of BATF leads, as expected, to increased BATF binding in the vicinity of genes. Nonetheless, whether the binding impacts gene expression of those genes has not been directly assessed. Is there a correlation between differential BATF binding to genes and their differential expression upon BATF co-expression, particularly in those genes that are shared by both BATF and IRF4, as well as by BATF and NFAT1. Are genes bound by these pairs of TFs among those found to be differentially expressed? What is their function? Moreover, is differential accessibility directly associated with differential expression upon BATF-OE?

11. Whilst PD-1 expression is decreased in BATF- compared to pMIG-transduced CAR T cells (Fig 1G and 1J), the authors found no significant difference in the mRNA levels of PD-1 (Fig 4A, line 235). Could this hint at post-transcriptional changes induced by BATF overexpression? This discrepancy is not mentioned further in the paper, and could be an important factor to take into account when interpreting the rest of the data.

When analysing LNs and spleen in search for memory CAR T cells, only the 5 mice that were capable of rejecting the tumour were analysed (Fig 3C). Do mice that were not able to reject the tumour show lower levels of these CAR T cells in secondary lymphoid organs? Is there a correlation between the number of memory CAR T cells in secondary lymphoid organs and delay in tumour growth?
Another aspect to consider are the kinetics of memory CAR T cell development, which could be done by analysing the number of memory CAR T cells peripherally and in secondary lymphoid organs over time. Do mice that develop memory CAR T cells earlier in time also lead to earlier suppression of tumour growth? Could this be an early predictor of the success of these CAR T cells?

12. Does BATF overexpression promote effector function and survival of human T cells in a

human ACT model in mouse, or at least in vitro?

Minor points:
Fig. 3C - the x-axis label could benefit from the antigen being measured in addition to the identity of the fluorocrhome

Reviewer #3:
Remarks to the Author:
Seo at el. follow-up previous observations that the collaboration of Nfat:Ap1 is necessary for the differentiation of functional T cells. In the present study they used a reporter system and investigated several transcription factors for their ability to increase or attenuate the nfat:Ap1 axis. This led to the identification of Batf as a positive modulator of this interaction. The authors then investigated how Batf overexpression affects effector profiles and the function of CARs T cells in tumor settings.

The authors provide very interesting and possibly promising new insights into how Batf improves the function of CAR T cells, which should be reported in principle. Nevertheless, there are several major concerns regarding the proposed mechanisms and the wider implications of the results. i) So far, only one tumor model has been analyzed. It remains to be determined whether similar findings can be made for other tumors. ii) It remains unclear how these findings are related to findings made earlier in infections, where different observations for Baft were reported. iii) As will be discussed in more detail below, the cooperative role between Batf and Irf4 favored by the authors is difficult to reconcile as in both tumor findings, Irf4 overexpression appears to counteract the effects of Batf.

Major points

- Figure 2: The authors state that the Tcf-1 population declines in Figure 2H and I. More data points and corresponding statistics would be helpful to justify this conclusion.

- Figure 3: The increased tumor survival after transferring Batf3 transduced CAR T cells is interesting. However, it relates only to 5 out of 24 mice and it appears to be a singular observation. The authors should report the outcome from replicate experiments to sustain their conclusions.
- Related to this point, the manuscript lacks in general information on experimental repeats.

- Figure 3: The authors conclude that Batf overexpression improves cell survival after tumor removal. However, while Batf overexpression helps to gain control over the tumor, it remains unresolved if the clearance of the tumor alone or the clearance of the tumor plus the Batf expression support the CAR maintenance. The earlier seems more like given the low Thy1.1 (BV711) expression levels in in Figure 3C (only 16 or 25% in Spleen and lymph-nodes). If Batf would be needed for the survival, one would expect a much higher if not close to 100% rate.

- The data presented in Figure 5 and the related conclusions are confusing. The authors favor that Batf needs to interact with IRF4 to boost the function of the CAR cells. However, there rather seems to be a survival issue as cells expressing mutant Batf are massively

lost. Figure 5F shows ~60% of CAR cells expressing Batf, 20% of cells express the empty vector control but only ~2% the Batf mutant. Accordingly, the Baft mutant has a detrimental impact on cell survival. Whether or not this survival defect involves Irf-4 or occurs through other mechanism remains unclear. This survival problem preclude making conclusion on whether or not the absence of Ifr4 interaction is needed for the increased CAR function after Batf transduction. Would a loss (or mutant form of Irf4) have the same impact?

- Figure 5 and S9: Overexpression of Ifr4 seems to attenuate the bosting effect of Batf overexpression (Figure S9B, S10H, 5D). How does this fit to the proposed collaborative role between the two TF? Moreover, the authors also conclude that Irf4 increases GranzymB expression but this increase is hard to be seen in in figure S9J, as there is no statistically significant difference in Figure S9J.

Minor points:

- Intro: It would be fair to also list the Immunity paper Utzschneyder et al. and Held, 2016 that initially reported on the role of the Tcf-1 population.

- Figure 1H: shows a clear reduction in PD-1 expression. To judge the remaining expression level, it would be very informative to have a reference of the unstained background level.

- The gating strategy in figure 2 remains unclear. Do the plots show all cells or are they gated on Batf positive cells?

- Figure 2: it remains unclear why the entire second figure is dedicated to illustrating mass-cytometry data which mainly reproduce what was already shown in figure 1. It is good data but could mostly be supplementary information.

- The authors state that KLRG-1 is upregulated following Batf transduction. The information is presently quite hidden in the presented datasets. To draw such a conclusion, a better representation of this findings should be presented.

- There is a typo in line 340 “Figure S96B-D”.

- The illustration of the experimental system, in particular what “bZIP” could be better explained for less informed readers.

Author Rebuttal to Initial comments

Responses to reviewers / Seo *et al* / NI-A30443-T, "BATF and IRF4 cooperate to counter exhaustion in tumour-infiltrating CAR T cells"

We appreciate the reviewers’ thoughtful and constructive comments, and we are heartened that all the reviewers see our findings as promising and useful to the field.

We have addressed the reviewers’ concerns with new experiments and additional analysis. The main additions to the revised manuscript are

// ChIP-seq and RNA-seq data and analysis for CAR T cells overexpressing BATF-HKE

// Bioinformatic analysis of the correlation between BATF binding and changes in chromatin accessibility

// Bioinformatic analysis of the binding sites targeted by BATF and IRF4, and the relation between transcription factor binding and changes in gene expression

// Replication of the experiments on BATF-overexpressing CAR T cells in B16 tumors

// New data showing the similar effects of BATF overexpression in an MC38 tumor model

// New data showing that BATF overexpression has similar effects in human CAR T cells

// A more thorough explanation of the evidence pointing to BATF-IRF cooperation.

The new bioinformatic analyses have identified several key elements influencing BATF-overexpression-induced $CD8^+$ TIL function: the early differential expression of *Tbx21*, *Eomes*, and other functionally relevant genes in the CAR T cells prior to adoptive transfer; alterations in signalling leading to less upregulation of IRF4 in response to TCR stimulation; a consequent redistribution of IRF4 among its target sites in chromatin; blunted TCR signalling to chromatin in the tumor, with a failure to open many exhaustion-related chromatin regions that normally become accessible in $CD8^+$ TILs; and a failure of the sustained upregulation of *Tox* that ordinarily occurs in the tumor.

The revised manuscript underscores both the specific point that BATF overexpression may be an option for improving the anti-tumor responses of CAR TILs, and the more general point that targeting T cell transcriptional programs can elicit coordinated changes in inhibitory 'checkpoint' receptor expression and in cellular signalling, and thus could serve as a complement to immune checkpoint therapies.

Point-by-point responses on all the issues raised by the reviewers are given below.

Reviewer #1

The manuscript by the Rao and Hogan labs is focused on the transcriptional programming of tumor infiltrating CAR-T cells, specifically on mechanisms that can be used to overcome their hypo-sensitization (exhaustion) thereby enhancing their efficacy from a therapeutic standpoint. The problem is of considerable translational interest and also raises fundamental issues that pertain to negative feedback control of effector T cell responses. The manuscript generates clear insight into the translational aspect but does not sufficiently explore the fundamental issues.
The authors begin by following up on earlier work which showed that CD8 CAR-T cells which are deleted for Tox or Nr4a family transcription factors, exhibit increased effector function and enhanced anti-tumor responses. Candidate transcription factors whose expression is upregulated by deletion of Tox or Nr4a genes are tested individually, using a gain-of-function approach to determine which can increase the effector functions of CAR-T CD8 cells in mouse models. This results in the main finding that BATF overexpression in CD8 CAR-T cells promotes their survival and expansion in the tumor microenvironment, increases production of effector cytokines, diminishes expression of inhibitory receptors and leads to the generation of long-lived memory T cells that can control recurrence of tumors. These findings are extensively documented and some are nicely extended to BATF over-expressing OT-I cells.
The remaining manuscript turns to the molecular mechanism(s) by which BATF over expression and importantly its sustained expression in tumor infiltrating CD8 T cells causes these phenotypic changes in the effector capacity of the cells and overcomes their propensity to undergo exhaustion. The most insightful experiment in this regard is the use of a BATF mutant protein which has previously been shown to be defective it its interaction with the transcription factor IRF4. This BATF mutant fails to enhance the expansion and effector capacity of the engineered CAR-T cells leading to the conclusion that BATF-IRF4 cooperate as molecular complexes to counteract CD8 T cell exhaustion.

From a mechanistic standpoint the following issues remain unresolved:

1. Which genes are directly co-targeted and co-regulated by BATF and IRF4 and likely account for the increased proliferative capacity of the CAR-T cells in the tumor microenvironment versus their enhanced effector functions and also the ability to generate memory. The RNA-seq and ChIP-seq experiments attempt to address these issues but do not analyze genes impacted by manipulating IRF4 levels. Furthermore, such target genes that are of strongest biological interest in the three fore-mentioned categories need to be highlighted.

We fully agree that the genes directly co-targeted and co-regulated by BATF and IRF4 are at the heart of the matter. A more detailed analysis of the RNA-seq data, as described in the revised text, has led us to distinguish between mRNAs already upregulated (or downregulated) in BATF-overexpressing cells at the time of T cell adoptive transfer, which could arguably poise the BATF-overexpressing cells to behave differently from control cells, and mRNAs upregulated (or downregulated) in the TILs *in vivo*, that implement the different behaviors of BATF-overexpressing and control cells upon encounter with the tumor.

The text and figures now highlight genes differentially expressed prior to adoptive transfer— for example, upregulated: *Tbx21*, *Il7r*, *Itgb7*, *Itgae*, *Icos*, *Slamf6*; downregulated: *Eomes*— that are likely to bias the response of BATF-overexpressing CAR T cells upon their initial encounter with the tumor and the tumor microenvironment. Separately, the text and figures highlight other genes differentially expressed in TILs (upregulated: *Ifnar1* and its downstream signalling effectors *Stat1* and *Stat3*; *Il12rb2*; downregulated: *Tox*) that may account for the enhanced effector function of BATF-overexpressing T cells in the tumor, and the ability to generate memory CAR T cells. Note that the latter genes are only differentially expressed between BATF-overexpressing and control cells after the cells encounter the tumor. The genes mentioned here are selected examples— a full listing is in TABLES S2 and S5.

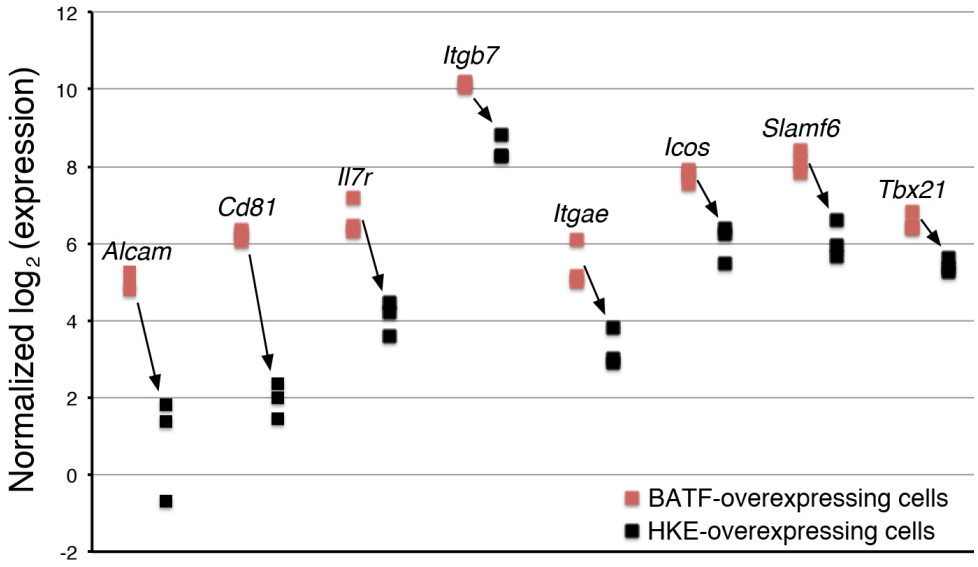
Whereas many of the genes differentially expressed at higher levels in BATF-overexpressing cells *in vitro* may be direct targets of BATF-IRF4, the genes that later implement the biological effects might be either direct targets or indirect targets that depend on BATF-elicited changes in cellular signalling pathways or transcription factors other than BATF-IRF4.

Genes that fail to be upregulated, or that are only weakly upregulated, in BATF-overexpressing TILs are an important part of the story. *Tox* [Figure 1I-J, 2D, 2I] is a compelling case, given its crucial role in exhaustion. Our ATAC-seq analysis indicates that TCR signalling to the *Tox* locus and other exhaustion-related accessible sites in chromatin is blunted in BATF-overexpressing TILs [Figure 5C-F], which is a telling observation given the evidence that continuing TCR signalling is a necessary precondition for the development of T cell exhaustion. We consider it very likely that the weak upregulation of *Tox* in BATF-overexpressing cells results secondarily from the modification of cellular signalling pathways, and not from direct targeting of the *Tox* gene by BATF-IRF4.

Regarding manipulating IRF4 levels: There were practical arguments against attempting to manipulate IRF4 protein levels directly. First, IRF4 binding at composite BATF-IRF sites was nearly maximal at endogenous IRF4 levels [Figure 6B], indicating that overexpression of IRF4 was unlikely to be informative; and, second, a knockdown strategy could not give a well-controlled low level of IRF4, since T cell stimulation itself increases IRF4 levels. We took the alternative approach of manipulating the BATF-IRF4 interaction with the HKE mutations. Overexpressing BATF-HKE was successful in lowering IRF4 binding at composite BATF-IRF4 sites across the board [Figure 6B], even below IRF4 binding in control pMIG cells, presumably by replacing endogenous BATF with the overexpressed BATF-HKE. Comparing the RNA-seq data from BATF-overexpressing cells and BATF-HKE-overexpressing cells confirmed that

BATF-IRF4 cooperation contributes to inducing the genes differentially upregulated in BATF-overexpressing cells at the time of adoptive transfer. Examples are in **REVIEWER FIGURE 1** (see further discussion in the response to Reviewer #2, comment 10, below).

2. Additional experiments with the BATF mutant protein would enhance mechanistic insight. This would involve performing ChIP-seq and RNA-seq experiments with such engineered TILs (earlier timepoints after adoptive transfer) to determine if the mutant BATF protein fails to bind to many or all sites that IRF4 is bound to or perhaps the mutant protein causes diminished IRF4 binding. This could then be connected to the alterations in gene expression. This experiment is particularly important as the authors suggest that endogenous BATF is sufficient to enable IRF4 binding to regions that contain AICE motifs recognized by BATF-IRF4 complexes.



REVIEWER FIGURE 1. Expression of selected mRNAs in BATF-overexpressing cells and BATF-HKE-overexpressing cells at day 6 *in vitro*. The examples shown were all upregulated in BATF-overexpressing cells compared to pMIG control cells, and, as is typical of this class of mRNAs, were expressed at lower levels in BATF-HKE-

Continuing the response to comment (1), above, we have carried out ChIP-seq and RNA-seq analyses of engineered cells expressing BATF-HKE, in parallel with ChIP-seq and RNA-seq analyses of BATF-overexpressing and pMIG control cells, all harvested after expansion *in vitro* at the point when they were ready for adoptive transfer. There are not enough TILs available for ChIP-seq after adoptive transfer.

The ChIP-seq experiments showed that BATF-HKE preferentially occupies the same sites as wild-type BATF. The level of BATF binding is substantially increased over that in pMIG control cells, but recruitment of IRF4 is diminished [Figure S10D, Figure 6B]. (Note that IRF4 binds almost exclusively at BATF sites [Figure 6A].) As noted in the response to comment (1), the diminished recruitment of IRF4 can be explained if BATF-HKE replaces endogenous BATF at the composite BATF-IRF sites. Some IRF4 binding is still observed, consistent with published evidence that BATF-HKE can cooperate with IRF4 on certain strong BATF-IRF composite elements in Th2 cells (Iwata et al, 2017).

Consistent with the diminished IRF4 binding, BATF-HKE cells do not express the genes that are significantly upregulated in BATF cells v pMIG cells at the same high levels (see the response to Reviewer #2, comment 10, below). The ChIP-seq and RNA-seq data for BATF-HKE are reported in the

GEO submission linked to the manuscript for readers who wish to analyze the data further. Since BATF-HKE-overexpressing cells have deviated already from BATF-overexpressing cells and pMIG cells at the time of adoptive transfer, and the cells recapitulate in tumors a phenotype— inability to expand and differentiate— that is familiar from the literature on BATF$^{-/-}$ CD8^{+} effector cells, we subsequently focused our attention on the differences between BATF-overexpressing cells and pMIG cells.

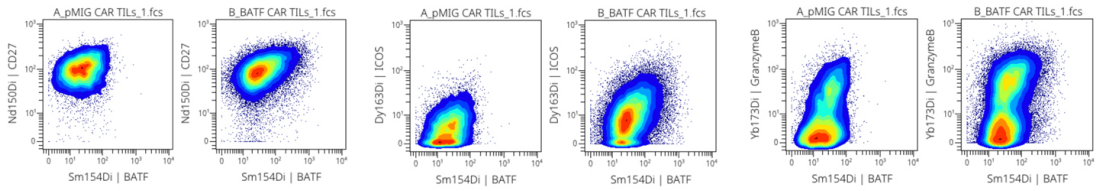
We would have liked to carry out the further experiments proposed by the reviewer, taking engineered BATF-HKE TILs from tumors for ChIP-seq and RNA-seq analyses. However, the experiments were not practicable, because recovery of BATF-HKE TILs from the tumors was too low, even at the earliest time points after adoptive transfer.

From a biological and therefore more fundamental standpoints the following issues remain to be addressed:

1. Is it increased expression of BATF or its sustained expression in CAR-T cells that results in their enhanced proliferation and effector functions? Does altering the levels of ectopically expressed BATF affect the various phenotypic properties?

We agree that the relation between BATF overexpression and the proliferation, survival, effector phenotypes of TILs is an important issue. We re-analyzed our flow cytometry data and CyTOF data, stratifying based on BATF expression level, to see whether the cellular phenotype is sensitive to BATF expression level or not. Indeed, there is a clear correlation between BATF protein level and CD27, ICOS, and granzyme B protein levels, already in evidence in pMIG cells, and more prominent in BATF-overexpressing cells [**REVIEWER FIGURE 2**]. These data suggest that the level of BATF protein could determine the strength of effector function, even in cells engineered to express unnaturally high levels of BATF.

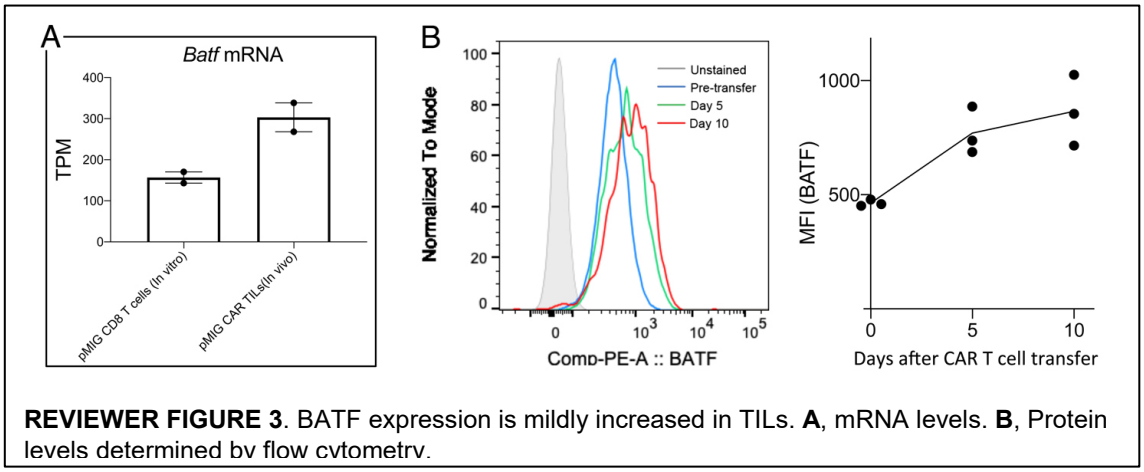
Regarding the issue of increased or sustained BATF expression, see the response to comment (3), below.



REVIEWER FIGURE 2. CyTOF analysis of pMIG control and BATF-overexpressing CAR TILs, illustrating the correlation between BATF protein level and CD27, ICOS, and Granzyme B protein

2. Can induction of BATF in exhausted CAR-T cells revive their proliferative and effector capacities? Is the exhausted state of a TIL reversed by BATF from an epigenetic and transcriptional standpoint?

The question whether manipulations that block the onset of exhaustion can also reverse established exhaustion has been a major outstanding question in CD8^{+} T cell biology for several years. For example, the question has not been resolved yet for NR4A and TOX proteins, despite the evidence from several laboratories that prior depletion of these proteins affords protection against the onset of exhaustion. Because it is a distinct issue that will require generation of new inducible models extensive new experimentation, we believe it is best reserved for a separate future study.



REVIEWER FIGURE 3. BATF expression is mildly increased in TILs. **A**, mRNA levels. **B**, Protein levels determined by flow cytometry.

3. What is the molecular mechanism by which BATF is repressed in exhausted CD8 T cells such that continued TCR signaling resulting in the activation of NFAT but not AP-1 fails to induce BATF? The direct dependency for its repression by Tox and/or Nr4a family members is not explored.

Although the possible regulation of *BATF* by NFAT, TOX, and NR4A proteins is is a matter worth pursuing, the question as posed starts from the premise that *BATF* is repressed in exhausted CD8⁺ T cells. It turns out that this is not the case.

Batf expression is elevated in engineered NR4A-depleted or TOX-depleted TILs compared to wild-type TILs (Chen *et al*, ref 5; Seo *et al*, ref 6), but this observation does not imply that *Batf* expression has gone down over time in wild-type TILs. In fact, RNA-seq experiments comparing control CD8⁺ T cells expanded *in vitro* under our conditions and day 8 CAR TILs from our experiments showed that there is a modestly higher level of *Batf* mRNA in the TILs [**REVIEWER FIGURE 3A**].

We had also observed no significant difference in *Batf* mRNA between exhausted OT-I T cells and bystander P14 T cells in the same tumor (Mognol *et al*, ref 28). To evaluate the underlying premise rigorously, we assessed BATF protein levels, pre-transfer and in CD8⁺ TILs at day 5 and day 10 after transfer, to determine whether endogenous BATF protein was repressed during the exhaustion process [**REVIEWER FIGURE 3B**]. The experiments confirmed the RNA-seq results: BATF protein in CAR TILs was slightly increased compared to pre-transfer CAR T cells.

For the specific question about NFAT, the data are in agreement with our separate findings that NFAT in the absence of AP1 does not cause a strong upregulation of *Batf*. In two independent series of experiments (Martinez *et al*, ref 12; P Ramchandani and PG Hogan, unpublished), expressing constitutively active NFAT1-RIT in CD8⁺ T cells elicited only ~1.3-fold increase (not statistically significant) in *Batf* mRNA expression.

Reviewer #2

Much attention is now being focused on the function of basic leucine zipper (bZip) transcription factors of the AP-1 family in regulating CD8+ T cell responses to chronic stimuli. Indeed the authors' earlier work has shown that while the TF NFAT plays a critical role in driving both T cell activation and exhaustion, the relative extent to which it drives either programme is affected by the levels to which NFAT is accompanied by its dimerisation partner, the AP-1 TF c-

Jun (Martinez et al., Immunity 2015). As a consequence, it is now known that overexpression of c-Jun augments CD8+ T cell function upon adoptive immunotherapy, by limiting exhaustion (Lynn et al., Nature 2019).

This manuscript by Seo et al. extends this earlier work, examining the ability of various AP-1 factors when overexpressed to augment anti-tumour immunity upon adoptive immunotherapy. They find that a different bZip TF, BATF, when overexpressed in CD8+ T cells augments their ability to mediate anti-tumour immune responses upon adoptive immunotherapy using two mouse models - a anti-CD19 CAR transduction / B16-hCD19 solid tumour model and the OT-1 TCR-tg / B16-Ova models and is superior to overexpression of Jun TFs, leading to striking tumour rejection.

The significance of the work should be considered in the context of prior work in this area: The relationship between BATF and CD8+ T cell function has been examined in several prior studies, each with somewhat different conclusions. Originally, it was proposed that BATF drives T cell exhaustion (Quigley Nat Med 2010). However, it has also been shown using gene knockout studies in mice that BATF is required for acute expansion of CD8+ T cells early following T cell stimulation (Kurachi et al., Nat Immunol 2014). BATF is known to participate in IRF4/JunD/BATF heterotrimers at DNA whose differential affinity for distinct enhancer sequences determines the extent to which weak TCR signals drive gene expression (Iwata et al., Nat Immunol 2017). Finally, it has been shown that REGNASE-1 deletion in CD8+ T cells in adoptive immunotherapy dramatically improves anti-tumour efficacy in a manner partially dependent upon increased BATF expression, since combined loss of BATF and REGNASE-1 reverse the phenotype resulting from REGNASE-1 deficiency alone (Wei et al., Nature 2019).

Regardless of these prior findings, however, the value of the work is that it defines a novel way to augment the anti-tumour function of CD8+ T cells and in the models used, this appears to be superior to the effect of c-Jun overexpression which has been the subject of quite intense interest. Thus, the manuscript is of interest to the field. However, the manuscript is rather correlative in nature, and a general model of how BATF promotes CD8+ T cell function in the context of adoptive immunotherapy does not clearly emerge from the data presented. In addition, while it is proposed that reduction in the exhausted state of CD8 T cells is a mechanism by which BATF overexpression augments T cell survival and immunotherapy, this has not been experimentally demonstrated.

Major comments:

1. BATF overexpressing cells have superior survival and effector function upon adoptive immunotherapy, and the authors correlate these observations with decreased expression of exhaustion markers, including PD-1, and TOX. It is therefore proposed that BATF functions by limiting exhaustion.The authors would need to justify these comments experimentally - ie is the phenotype (increased survival) reversed upon restoration of PD-1 expression, or phenocopied (in terms of T cell survival and anti-tumour efficacy) upon genetic ablation of PD-1 or TOX? These are important experiments especially given the authors observe decreased TOX levels in BATF-overexpressing cells. Loss of TOX expression has previously been shown to result in decreased CD8+ T cell survival (Scott et al., Nature 2019). How do the authors reconcile these observations?

We did not propose in the original manuscript that overexpressing BATF limits T cell exhaustion. Rather, our conclusion was that the engineered BATF-overexpressing cells are diverted to an alternative fate, with a distinctive effector-like phenotype, which provides more effective control of tumor growth. This is still the principal conclusion of the revised manuscript. However, our new detailed analysis of ATAC-seq data has made it clear that there is differential opening in pMIG control cells of chromatin regions that we have previously characterized as responding to TCR stimulation in the tumor (Mognol *et al*, ref 28) [Figure 5C-E], including 'exhaustion-specific' regions, so in that sense the development of the exhaustion program is blunted in BATF-overexpressing cells. We do not believe that the blunting of TCR-dependent chromatin opening in BATF-overexpressing cells is necessarily a direct effect of BATF on the loci surveyed. Rather, it is likely to be a secondary consequence of cellular adaptations to BATF

overexpression, altering other signalling proteins and transcription factors and blunting both activation and exhaustion responses.

On the question of manipulating PD1, TOX, and other exhaustion-related genes: BATF overexpression alters the expression of many genes, as we have demonstrated. The observed changes in PD1 and TOX protein expression in the population do not mean that BATF overexpression acts directly to repress *Pdcd1* or *Tox* transcription, or that BATF overexpression improves anti-tumor responses primarily via changes in PD1 and TOX protein levels. Rather, we would argue from our ATAC-seq data that attenuated signalling downstream of the TCR could account for the coordinate changes in inhibitory receptors and the diminished upregulation of TOX. Experimentally altering PD1 or TOX levels would be predicted to have effects on effector function whether or not the two proteins are implicated in the primary action of overexpressed BATF. For this reason, we consider it unlikely that manipulating PD1 or TOX levels in the context of BATF overexpression would be informative.

In regard to the reduced level of TOX and its implications for $CD8^+$ TIL survival: What we see in BATF-overexpressing T cells is not loss of TOX expression. It is a failure to upregulate TOX to the same extent as in control cells, and this partial failure is likely to be beneficial to CAR T cell function by disfavoring the progression to exhaustion. Our own work and reports from other groups document that cells with reduced expression of TOX (achieved by shRNA knockdown or use of $Tox^{+/-}$ cells) exhibit elevated antitumor function compared to $CD8^+$ TILs that express TOX at high levels (Seo *et al*, ref 6; Wang *et al*, ref 8; Khan *et al*, ref 10). It is only the complete absence of TOX in $Tox^{-/-}$ cells that leads to a failure to survive long-term in the tumor. We and others have interpreted the results as implying that a modicum of TOX expression is necessary for the maintenance of functional $CD8^+$ TILs, but the elevated TOX expression characteristic of unmanipulated $Tox^{+/+}$ TILs induces exhaustion and impairs effector function.

2. The authors see loss of memory-associated molecules including TCF-1, CD62L and CD127 and express increased expression of KLRG-1, associated with terminal differentiation, at early timepoints - observations that are somewhat difficult to reconcile with the superior survival of BATF-overexpressing cells. At late timepoints, increased frequencies of memory cells are observed.

The BATF-overexpressing TILs are a heterogeneous collection comprising different subsets of cells. The cited differences represent an average over the entire population. There is not a thoroughgoing loss of TCF1, CD62L, and CD127, nor is there uniform expression of KLRG1. Large numbers of cells still show high expression of TCF1, CD62L, and CD127. Only a few express high levels of KLRG1. The findings are therefore not at variance with the observed survival of BATF-overexpressing cells.

2, continued. How does BATF promote increased survival of T cell responses? Can the authors propose a model, justified by new experimental data, for how BATF overexpression promotes CD8+ T cell survival and effector function? Is it primarily through driving a larger acute expansion phase of the response? Is this function independent of phenotype, and is it primarily through increased proliferation or decreased apoptosis?

We proposed a first-order model based on our experimental data in the original manuscript. Our model was that BATF-IRF cooperation is required for CAR TIL survival and effector function. The model is consistent with previous work documenting that BATF and IRF4 are independently required for the differentiation of effector $CD8^+$ T cells (Kurachi *et al*, ref 20; Man *et al*, ref 26; Man *et al*, ref 49), but it is new in pinpointing the role of the BATF-IRF interaction in $CD8^+$ T cells, and specifically in $CD8^+$ TILs. The large increase in cell numbers can be accounted for by increased proliferation (see our response to

comment (2), below), but, in addition, the increased ability to eradicate tumor cells is almost certainly related to the enhanced effector phenotype of the CAR T cells.

To fill in more mechanistic detail and identify genes associated distinctively with BATF overexpression in $CD8^+$ T cells, we have conducted an extensive bioinformatic analysis of the data. As specified in our response to comment (1) of Reviewer #1, we now discriminate between differential early transcriptional changes caused by BATF overexpression or by the combination of BATF overexpression and acute activation *in vitro*, and transcriptional differences emerging subsequently within the tumor microenvironment. In the revised text and figures we have pointed to some genes that could figure in the initial response upon encountering the tumor and in the later progression of responses within the tumor. Data for all genes expressed at the time of adoptive transfer and genes expressed in TILs recovered from tumors are presented in TABLES S2 and S5 and in the GEO submission linked to the manuscript.

The BATF-IRF interaction is necessary for effective tumor clearance under our conditions, reminiscent of findings in the literature that BATF and IRF4 are essential for $CD8^+$ T effector differentiation and expansion in acute infections. The increased occupancy of AP1-IRF composite element (AICE) sites by BATF and the apparent redistribution of IRF4 among those sites— both of which we have demonstrated experimentally— could well account for the increased effector function of BATF-overexpressing CAR TILs. As we mentioned in the original Discussion, our experiments leave open the possibility that a separate class of non-AICE BATF-binding sites is required for the maintenance of 'progenitor' $CD8^+$ T cells responsible for memory formation. Indeed, the increased expression of ETS-1 in the engineered CAR T cells opens the possibility that functional cooperation between BATF and ETS-1 has a key role in maintaining the progenitor population, as previously shown in differentiation of $CD4^+$ T cells (Pham *et al*, ref 50).

2, continued. Whilst they use Ki67 staining for showing that there is an increase in CAR T cell proliferation, the median increase is of 1.5 fold (Fig 1J). Is such an increase enough to allow for the huge increase in survival?

Ki67 staining is an imperfect proxy for rate of cell proliferation, but the short answer is yes, this could in principle account for the increase in cell numbers. (If the brightest cells in the Ki67 plot of Figure S2C represent the fraction in cell cycle phases G2 and M— the time of maximal Ki67 protein levels— a larger fraction implies briefer G1 and therefore a faster cell cycle. Because cell number increases exponentially with time, even a 30% decrease in length of the cell cycle could account for the observed difference in cell numbers.) We do not claim that this is the only factor at work, since we have not quantitated initial homing of CAR T cells to the tumor, and have not attempted to measure differences in cell death in the tumor environment.

3. The reporter system to test the activity of bZip TFs of interest in driving gene expression by NFAT:AP-1 sites (Fig S1A-F) utilised a retroviral vector with tandem NFAT:AP-1 sites driving expression of both Thy1.1 and the bZip TF whose function was being tested - resulting in a positive feedback loop as the authors state. This feedback loop precludes straightforward interpretation of the experimental results. A simpler reporter system should be developed where a constitutive promoter drives expression of the bZip TF of interest allowing the activity of different TFs to be tested in a fair comparison absent of such positive feedback artefact.

In our view, the experiments in Figure 1 meet the request for a 'simple' overexpression of BATF, JUN, and MAFF in CAR T cells. Other bZIP factors could also be tested in this way, but those experiments would be beyond the scope of this manuscript.

4. Fig. 1. Is the expression (in terms of MFI or percentage expression) of the transduced CAR affected by the co-transduction with the various bZip factors? Data could be shown

Expression of the CAR assessed by MFI was not affected, and CAR transduction efficiency was always over than 90%, no matter which bZIP construct was co-transduced. We have added the data as Figure S2C.

5. Fig. 3B. It is known that inoculation of killed B16 cells generates immunity to further B16 administration, so it is not clear from the data presented that the surviving T cell response drives de novo rejection of the B16-Ova secondary tumour. A good control would be to implant either B16-Ova or B16 control lines into animals that rejected the initial B16-Ova tumours to demonstrate that it is the Ova-specific response that caused rejection of the tumours.

We agree that it is an open question whether memory CAR T cells alone account for rejection of the second tumor. Operationally, for CAR T cell immunotherapy, it is beneficial whether memory CAR T cells kill the tumor by themselves, or CAR T cells (in the process of eliminating the first tumor) educate the immune system to recognize and reject the second tumor. The [REDACTED].

Meanwhile, we believe our current data will be of substantial interest to the CAR T cell field in and of themselves. We have shown that BATF-overexpressing memory CAR T cells persist in the spleen and lymph node— in large numbers in some mice— after killing the B16-hCD19 tumor. They are plausible candidates to lead the attack on the second implanted tumor. If the attack is augmented by endogenous memory CD8 T cells spawned by the CAR T cell attack on the initial tumor, so much the better. We have clarified that both possibilities should be considered at this point.

6. Fig. S4D. The accessibility of BATF-bound sites at the TOX locus was decreased in the absence of BATF. Is this locus specific? What is the general function of BATF in regulating chromatin accessibility at its binding sites?

With regard to the *Tox* locus specifically, the differential accessibility should be construed as a failure to open efficiently upon exposure to the tumor, not as a decrease in accessibility [Figure 5F]. Other TIL 'exhaustion-specific' and 'activation-specific' regions display the same effect [Figure 5C-D]. As stated above in the response to comment (1) of this reviewer, we do not consider this to be a direct effect of BATF.

More generally, the comment alludes to the pioneer function attributed to BATF in other studies (Ciofani *et al*, ref 23; Kurachi *et al*, ref 20; Li and Leonard (2018) *Front Immunol* 9:2738; Pham *et al*, ref 50. It is not our immediate goal to establish a pioneer function for BATF in $CD8^+$ T cells, but we have examined whether there is a strong correlation between BATF site occupancy and increased chromatin accessibility in BATF-overexpressing cells. In brief, we were able to demonstrate a statistically significant correlation for a subset of sites that are poorly accessible in pMIG control cells. This might suggest that BATF itself drives the process of chromatin rearrangement, and the specific chromatin regions identified in TABLE S4 could form the basis for future investigations in this system.

6, continued. Can bioinformatic analysis of ATAC-Seq data at BATF sites be performed to examine whether BATF-bound sites generally increase or decrease in occupancy when BATF is overexpressed?

BATF occupancy assessed by ChIP-seq increases uniformly when BATF is overexpressed [Figure S10A]. We expect that the increase comes from a combination of BATF binding to sites that were incompletely occupied in pMIG cells and replacement of endogenous BATF and BATF3 at sites where either BATF or BATF3, or both, can bind in control pMIG cells.

Considering all accessible regions defined by ATAC-seq, only 2% were scored as differentially accessible in BATF-overexpressing cells compared to pMIG cells [Figure 5B]. This is likely due to the fact that

retroviral transduction requires prior activation through the TCR, and this activation by itself will upregulate both BATF and IRF4/IRF8 at least transiently in pMIG cells [Figure 6F, Figure S13], so that accessibility changes in many chromatin regions where BATF can act as a pioneer factor may have been accomplished already in pMIG cells. Of the differentially accessible regions, 551/640 overlap a BATF ChIP-seq peak, but further experiments would be needed to determine whether BATF binding contributes to increased accessibility or increased accessibility contributes to BATF binding. Focusing just on BATF binding sites identified by ChIP-seq, chromatin accessibility at the vast majority does not change appreciably when BATF is overexpressed. Accessibility does increase substantially at a small minority of BATF sites that are poorly accessible in pMIG control cells. We have added this analysis in the revised manuscript [Figure 7A].

7. When BATF is overexpressed, is the binding of another TF reduced, whose function is to subvert T cell effector function, promote exhaustion marker expression and limit survival? Ie is a competition model with another bZip TF most relevant - in which case, can ChIP-Seq experiments be performed to examine what factors bind less when BATF is overexpressed? Or is NFAT binding alone in the absence of BATF? Can this be experimentally demonstrated? Can ATAC-footprinting be informative here?

Physical competition with another transcription factor is a conceivable mechanism by which BATF overexpression could enforce changes in TIL transcription and biology. However, the positive outcomes of BATF overexpression cited— increased effector function, decreased exhaustion marker expression, and enhanced survival— are not necessarily conferred by the effect of BATF on expression or binding of a single transcription factor, and competition is only one conceivable mechanism by which BATF could act. It would not be efficient to examine transcription factors one by one at this point, nor is it technically feasible to perform ChIP-seq for a panel of candidate transcription factors in TILs, where cell numbers are limiting. To capture an unbiased picture of the genes upregulated or downregulated by BATF overexpression, we have focused on RNA-seq as the logical first level of analysis, and carried out the analysis both pre-adoptive transfer and after CAR TIL encounter with the tumor [Figure 5A, 6C-E; TABLES S2 and S5; and GEO submission linked to the manuscript]. Genes detected as differentially expressed in the RNA-seq experiments will of course include genes directly regulated by BATF, genes that are controlled indirectly by BATF regardless of the specific mechanism, and any genes that are targets for competition between BATF and another transcription factor. The next level of analysis, in a future study, will parse the detailed mechanisms, including possible BATF competition with other transcription factors.

8. Fig. 5. Whilst there is a clear difference between the outcome of BATF and BATF-HKE overexpression, further experiments to confirm the hypothesis disruption of BATF and IRF4 binding is "absolutely required" would be necessary, particularly since this is one of the main findings and thrust of the paper. Is it possible that the HKE mutation, under overexpression conditions, could disrupt some other function of BATF other than the IRF4 interaction?

There is little reason to propose that BATF-HKE gains a disruptive function. BATF knockout CAR T cells replicate the findings for BATF-HKE CAR T cells. They fail to accumulate in the tumor, and the few cells recovered from the tumor show the same dearth of $PD1^{+}TIM3^{+}$ cells and the same preponderance of $TCF1^{+}$ cells. So a simple loss of function model is sufficient to explain the findings.

Moreover, IRF4 binds almost exclusively at sites shared with BATF [Figure 6A], and our new ChIP-seq data for IRF4 [Figure 6B], cited in a response to Reviewer #1, indicate that binding of IRF4 at BATF-IRF composite sites is much reduced in the BATF-HKE-overexpressing cells. The parsimonious interpretation

is that BATF-HKE acts as a partial loss-of-function mutant, and that the function that is lost is the ability to recruit IRF4 (and potentially IRF8) to the composite sites. The literature has documented a similar mechanism in other T cell contexts— for example, BATF has been shown to act in $CD4^+$ Th2 and Th17 cells largely through recruiting IRF4 to AICE elements (Murphy *et al*, ref 21; Li *et al*, ref 22; Glasmacher *et al*, ref 24; Iwata *et al*, ref 37). This is however a completely novel finding for $CD8^+$ T cells, and specifically for $CD8^+$ TILs.

8, continued. It could be of interest to test whether cells carrying IRF4 mutations (instead of BATF mutants) unable to form the BATF-IRF4 association would lead to the same outcome as BATF-HKE mutants.

If there were a known IRF4 mutant with the specified properties, we would have tested its effects. In fact, no such mutant has been reported, despite the use of BATF-HKE mutant to interrogate the BATF-IRF interactions over a period of several years. It would be a prohibitive task to design and validate such an IRF4 mutation within a reasonable time frame.

8, continued. As a control, the authors should test whether the HKE mutations in BATF result in its diminished expression or DNA binding in T cells - these are important controls in interpreting the profound loss of function when BATF-HKE is overexpressed. Only in the case that these are unaffected can it be stated that the interaction with IRF4 is necessary for the observed function.

We compared BATF and BATF-HKE expression at the time of adoptive transfer in the original manuscript, in Figure 5A, and found that BATF-HKE is overexpressed at comparable levels to wildtype BATF. New data showing that BATF-HKE DNA binding at BATF sites is roughly comparable to binding of wildtype BATF, and that IRF4 binding is substantially reduced at the majority of sites occupied by BATF-HKE, is provided in the revised manuscript [Figure 6B, S10C].

8, continued. It may be interesting to study differences in the transcriptome upon overexpression of BATF and BATF-HKE, as this could provide evidence of how the HKE mutants differ from BATF-transduced cells. Is there a decrease in the transcription levels of IRF4-bound genes in HKE mutants compared to BATF CAR T cells?

We have added RNA-seq data for parallel samples of cells overexpressing BATF-HKE and wild-type BATF to the revised manuscript [Figure S10F] and to the GEO submission linked to the manuscript. In brief, expression of the BATF-IRF target genes identified as differentially expressed in BATF v pMIG cells is reduced in BATF-HKE-overexpressing cells compared to BATF-overexpressing cells (see **REVIEWER FIGURE 1**, and response to comment (10) below).

9. BATF WT and HKE overexpressing cells both diminished PD-1 expression but had divergent effects on survival - what does this mean? Does it mean that the function of BATF WT in promoting survival has nothing to do with its effect on PD-1 expression?

Both BATF and BATF-HKE diminished PD-1 expression, but probably for different reasons, diversion to an alternative fate in the first case, and a failure of T cell activation and differentiation in the second. We are not proposing that BATF overexpression has its principal effects on CAR TIL function through the rather modest reduction in PD1 levels.

9, continued. Effects of PD-1 on survival could be discussed in the context of Scott et al. (Nature, 2019) and Odorizzi et al. (JEM, 2015), where loss of exhaustion marker expression is associated with diminished - not enhanced - survival.

We can include a discussion of the effects of PD1 on survival in the data of Scott *et al* and Odorizzi *et al* if the editors and reviewers believe it will be useful.

10. ChIP analyses show that overexpression of BATF leads, as expected, to increased BATF binding in the vicinity of genes. Nonetheless, whether the binding impacts gene expression of those genes has not been directly assessed. Is there a correlation between differential BATF binding to genes and their differential expression upon BATF co-expression, particularly in those genes that are shared by both BATF and IRF4, as well as by BATF and NFAT1. Are genes bound by these pairs of TFs among those found to be differentially expressed? What is their function? Moreover, is differential accessibility directly associated with differential expression upon BATF-OE?

We have analyzed the RNA-seq data for a correlation between increased BATF-IRF4 binding and increased gene expression. There are clear examples of known BATF-IRF4 target genes (*Alcam*, *Ezh2*) that exhibit enhanced BATF-IRF4 binding and are differentially more expressed in BATF-overexpressing cells [Figure 7D], lending credence to the idea that there is a connection between differential binding of BATF-IRF4 and differential gene expression. Another test of the correlation between BATF-IRF4 binding and differential expression is whether BATF-HKE is less capable of causing expression of other candidate BATF-IRF4 target genes. Examining all 46 genes that were differentially expressed at higher levels in BATF-overexpressing cells v pMIG cells (*adj p* <0.05, and excluding *Batf* itself), 42 were expressed at lower levels in BATF-HKE-overexpressing cells than in BATF-overexpressing cells; and in 24 of those cases the difference between HKE cells and BATF cells was statistically significant (*adj p* <0.05). Examples are shown in **REVIEWER FIGURE 1**.

Nonetheless, we became skeptical of a lock-step correlation between observed gene expression and BATF-IRF4 binding as a result of several observations: (1) In cases like *Alcam* and *Ezh2*, the increase in IRF4 binding was only modest; (2) other genes exhibiting differential expression showed high occupancy of IRF4 peaks already in pMIG control cells, with little change or even a slight reduction of IRF4 binding in BATF-overexpressing cells; (3) the differences in IRF4 binding between BATF-overexpressing cells and pMIG control cells were modest across all BATF-IRF sites [Figure 7C]; and (4) even for the exemplary cases *Alcam* and *Ezh2*, mRNA levels changed appreciably upon αCD3/αCD28 stimulation, but not in the same direction [Figure 7D], indicating that transcription factors other than BATF and IRF4 might predominate in dictating the overall transcriptional outcome.

Our current view is that IRF4 recruitment by BATF has a positive role in expression of many of the differentially expressed genes, but that its role is obscured to an extent when comparing BATF-overexpressing cells to pMIG control cells, because there is already substantial IRF4 bound at the relevant sites in pMIG cells, most likely due to prior activation and BATF and IRF4/IRF8 induction as discussed above. The changes in BATF-IR4F binding induced by BATF overexpression can be expected to impose a bias on gene expression, but the bias acts in the context of other alterations in transcription factor levels and activation between BATF-overexpressing cells and pMIG control cells.

We have noted in the revised text some specific differentially expressed genes that are likely to contribute to the altered phenotype of BATF-overexpressing cells when they first encounter the tumor.

Regarding the final question in comment (10), we examined whether differential chromatin accessibility is directly reflected in differential gene expression and, if so, to what extent. There are relatively few significant increases in chromatin accessibility in BATF-overexpressing cells (only ~2% of total accessible regions detected, again most likely because of the prior activation) [Figure 5B], but they are often linked directly to BATF binding (551/640 regions of increased accessibility overlap with a BATF ChIP-seq peak).

In some of these cases we have documented an association of increased chromatin accessibility with increased gene expression [Figure 7A-B]. To enable further investigation, we provide a list of chromatin regions where strong differential BATF binding is associated with strong differential accessibility in TABLE S6.

11. Whilst PD-1 expression is decreased in BATF- compared to pMIG-transduced CAR T cells (Fig 1G and 1J), the authors found no significant difference in the mRNA levels of PD-1 (Fig 4A, line 235). Could this hint at post-transcriptional changes induced by BATF overexpression? This discrepancy is not mentioned further in the paper, and could be an important factor to take into account when interpreting the rest of the data.

The reviewer notes, correctly, that the average PD1 protein difference between the two populations is about 4-fold, whereas the difference in the mRNA levels of *Pdcd1* did not score as significant. This indicates a difference in the rates of new protein synthesis per mRNA molecule, of protein degradation, or both. We did not measure these parameters, and addressing them, while very interesting for a future study, is beyond the scope of the current manuscript.

11, continued. When analysing LNs and spleen in search for memory CAR T cells, only the 5 mice that were capable of rejecting the tumour were analysed (Fig 3C). Do mice that were not able to reject the tumour show lower levels of these CAR T cells in secondary lymphoid organs? Is there a correlation between the number of memory CAR T cells in secondary lymphoid organs and delay in tumour growth?

Under our experimental protocol, mice had to be sacrificed in those cases where BATF-overexpressing CAR T cells initially delayed tumor growth but then failed to control the tumor in the longer term, and therefore we were not able to assess the presence of memory CAR T cells in those mice many weeks later. Among the surviving mice in the initial experiment, there was some evidence suggesting a correlation, in that the mouse with the lowest frequency of CAR T cells in the spleen and draining lymph nodes was the sole mouse that showed any initial growth of the tumor implanted at day 120, but the sample size was too small to draw a firm conclusion.

We have since replicated the B16-hCD19 experiment with a similar outcome. In detail, 3/10 mice completely cleared the tumor. CAR T cells accounted for ~7% of $CD8^+$ T cells in spleen and LN of these mice, and the cells exhibited a memory phenotype ($CD44^{high}$, $CD62L^{high}$, $TCF1^{high}$), replicating the findings of the first experiment. When the three surviving mice were rechallenged, tumor growth was completely blocked in each case, again replicating the earlier findings.

11, continued. Another aspect to consider are the kinetics of memory CAR T cell development, which could be done by analysing the number of memory CAR T cells peripherally and in secondary lymphoid organs over time. Do mice that develop memory CAR T cells earlier in time also lead to earlier suppression of tumour growth? Could this be an early predictor of the success of these CAR T cells?

Relating to the early appearance of CAR T cells in lymph nodes, when we have analyzed the tumor-draining lymph node at early times after CAR T cell adoptive transfer, we have typically seen less than 0.2-0.3% CAR T cells. In contrast, 100 days after tumor clearance, we detected a median of ~20% memory CAR T cells in the draining lymph node and spleen after tumor rechallenge, with considerable variability from animal to animal, as reported in the original version of the manuscript. As for sampling secondary lymphoid organs over time prior to clearance of the first tumor or prior to rechallenge, without sacrificing the animals, our current animal protocol does not allow us to do this, and, given the time involved, we respectfully submit that this experiment is outside the scope of the current study.

12. Does BATF overexpression promote effector function and survival of human T cells in a human ACT model in mouse, or at least in vitro?

As with mouse T cells, BATF overexpression enhances the effector function of human T cells. We obtained a human CAR construct from Carl June and constructed a human BATF overexpression vector. We transduced human $CD8^+$ T cells with a human CAR construct, and with a human BATF overexpression vector or its control vector, cultured them together with hCD19-bearing tumor cells, and assessed function by measuring cytokine production, *in vitro* proliferation, and *in vitro* cytotoxicity [Figure S4]. The BATF-overexpressing CAR T cells exhibited improved effector function by all these measures.

Minor points: Fig. 3C - the x-axis label could benefit from the antigen being measured in addition to the identity of the fluorocrhome

We have added the label.

Reviewer #3

Seo at el. follow-up previous observations that the collaboration of Nfat:Ap1 is necessary for the differentiation of functional T cells. In the present study they used a reporter system and investigated several transcription factors for their ability to increase or attenuate the nfat:Ap1 axis. This led to the identification of Batf as a positive modulator of this interaction. The authors then investigated how Batf overexpression affects effector profiles and the function of CARs T cells in tumor settings.

The authors provide very interesting and possibly promising new insights into how Batf improves the function of CAR T cells, which should be reported in principle. Nevertheless, there are several major concerns regarding the proposed mechanisms and the wider implications of the results.

i) So far, only one tumor has been analyzed. It remains to be determined whether similar findings can be made for other tumors.

New experiments with an MC38-hCD19 colon adenocarcinoma model have supported the findings with the B16 melanoma model, and we have included the new data in the revised manuscript [Figure S2E]. The capsule summary: all ten mice implanted with the MC38 tumor showed a delay in tumor growth compared to controls, and 2/10 mice cleared the tumor completely.

ii) It remains unclear how these findings are related to findings made earlier in infections, where different observations for Batf were reported.

We agree that findings in the literature regarding BATF in infections are varied. In particular, two influential papers reported modest upregulation of BATF in chronic infections (Quigley *et al*, ref 27; Man *et al*, ref 26) which, together with some other observations, led to the notion that BATF might be a driver of exhaustion. We think it is more likely that BATF, like NFAT, can have an ambivalent role that depends on the context. A separate report for chronic LCMV clone 13 infection closely paralleled our findings, demonstrating that overexpressing BATF in virus-specific P14 TCR-transgenic $CD8^+$ T cells increased their proliferation, expression of effector markers, and control of the viral infection (Xin *et al*, ref 52). We relate these earlier findings to ours in the Discussion.

iii) As will be discussed in more detail below, the cooperative role between Batf and Irf4 favored by the authors is difficult to reconcile as in both tumor findings, Irf4 overexpression appears to counteract the effects of Batf.

The interpretation is more complex. We submit that IRF4 overexpression has little effect on the BATF-overexpression phenotype at early times. In original Figure S9F there was no significant difference between overexpression of BATF+IRF4 and overexpression of BATF alone in limiting tumor growth at day 20. Original Figures S9G and S9I showed that overexpression of BATF+IRF4 in fact increased the percentage of OT-I TILs compared to BATF alone in this case, and resulted in an equally low fraction of fully exhausted PD1^{+}TOX^{+} CAR T cells. In contrast, IRF4-overexpressing cells resembled control CAR T cells on these measures. It is difficult to argue from these findings that IRF4 was counteracting the effects of BATF at the 20-day time point.

There was a small but statistically significant effect on the early tumor growth in original Figures S9B and S9C, though it was really only after day 30 that the results for BATF+IRF4 overexpression and BATF overexpression diverged. Note, however, that adding BATF overexpression improved tumor control over IRF4 overexpression alone. We discuss further whether IRF4 should be viewed as counteracting the effects of BATF in the revised Discussion, and in our response to the reviewer's comment on Figure 5 and Figure S9, below.

Major points

- Figure 2: The authors state that the Tcf-1 population declines in Figure 2H and I. More data points and corresponding statistics would be helpful to justify this conclusion.

The CyTOF result in Figure 2 has also been observed in many flow cytometry experiments. We have added a typical flow cytometry plot for TCF1 in Figure S2F. It is important to note that the decline of TCF1 amounts to a modest quantitative difference, not a massive loss of TCF1^{+} cells. There are still clearly enough TCF1^{+} cells to maintain the population and to give rise to abundant memory CAR T cells in the majority of surviving mice. Please see also our response to comment (2) of Reviewer #2.

- Figure 3: The increased tumor survival after transferring Batf transduced CAR T cells is interesting. However, it relates only to 5 out of 24 mice and it appears to be a singular observation. The authors should report the outcome from replicate experiments to sustain their conclusions.
- Related to this point, the manuscript lacks in general information on experimental repeats.

The beneficial effect of transferring BATF-overexpressing CAR T cells was not restricted to 5/24 mice studied in the first experiment. Tumor growth was delayed in all the mice that received BATF-overexpressing CAR T cells, and survival was prolonged compared to controls for the entire cohort, as evidenced in the Kaplan-Meier plot [Figure 1D]. The findings have been further confirmed in a replicate B16-hCD19 experiment that is included in the revised manuscript [Figure S2D]. In detail, 3/10 mice completely cleared the tumor. CAR T cells accounted for ~10% of CD8^{+} T cells in spleen and LN of these mice, and the cells exhibited a memory phenotype (CD44^{high}, CD62L^{high}, TCF1^{high}) similar to that reported for the first experiment. Rechallenge tumor growth was completely blocked in all three surviving mice. Similar results have been obtained in an MC38 adenocarcinoma model [Figure S2E], as noted above.

The number of replicate experiments is noted in the figure legends and/or in Methods, as appropriate.

- Figure 3: The authors conclude that Batf overexpression improves cell survival after tumor removal. However, while Batf overexpression helps to gain control over the tumor, it remains unresolved if the clearance of the tumor alone or the clearance of the tumor plus the Batf expression support the CAR maintenance. The earlier seems more like given the low Thy1.1 (BV711) expression levels in in Figure 3C (only 16 or 25% in Spleen and lymph-nodes). If Batf would be needed for the survival, one would expect a much higher if not close to 100% rate.

We showed that BATF-overexpressing cells survived as memory CAR T cells after tumor clearance. We could not assess the survival of control CAR T cells in the absence of BATF overexpression, because control CAR T cells did not clear the tumor, and the mice had to be sacrificed. The salient point is precisely that the engineered memory CAR T cells are present long after the tumor is cleared.

In addition, perhaps the text explanation of Figure 3C was itself not crystal clear. Figure 3C quantitated CAR T cells as a fraction of total $CD8^+$ T cells, where the majority of the total $CD8^+$ T cell population is comprised of endogenous $CD8^+$ T cells, as expected. In fact, representation of CAR T cells at levels of 20% or more of total $CD8^+$ T cells several months after the primary tumor was cleared, and immediately after the rechallenge tumor was cleared, is noteworthy.

- The data presented in Figure 5 and the related conclusions are confusing. The authors favor that Batf needs to interact with IRF4 to boost the function of the CAR cells. However, there rather seems to be a survival issue as cells expressing mutant Batf are massively lost. Figure 5F shows ~60% of CAR cells expressing Batf, 20% of cells express the empty vector control but only ~2% the Batf mutant. Accordingly, the Batf mutant has a detrimental impact on cell survival. Whether or not this survival defect involves Irf-4 or occurs through other mechanism remains unclear. This survival problem preclude making conclusion on whether or not the absence of Ifr4 interaction is needed for the increased CAR function after Batf transduction. Would a loss (or mutant form of Irf4) have the same impact?

Indeed, Figure 5F shows that ~60% of TILs are CAR T cells in the case of BATF overexpression, -20% in the case of pMIG control, and ~2% in the case of BATF-HKE overexpression. However, in the case of BATF KO donor T cells, again only ~2% of TILs are CAR T cells. There is no need to postulate an additional detrimental impact of BATF-HKE, when simple absence of BATF from the CAR T cells is enough to produce the effect. The literature documents that BATF can act in Th2 and Th17 cells largely through recruiting IRF4 to AICE elements, and that the HKE mutations severely impair IRF4 recruitment (Murphy *et al*, ref 21; Li *et al*, ref 22; Glasmacher *et al*, ref 24; Iwata *et al*, ref 37). We have shown in the revised manuscript that nearly all IRF4 in $CD8^+$ T cells binds at BATF-IRF4 sites, and that BATF-HKE severely impairs that binding, indicating that the HKE mutation does have a deleterious effect on IRF4 binding and gene expression. The most straightforward view is that both manipulations of $CD8^+$ CAR T cells— knocking out BATF and overexpressing BATF carrying the HKE mutations— act by abrogating BATF-IRF-dependent transcription.

Loss of IRF4 is likewise known to impair metabolic reprogramming and clonal expansion of effector $CD8^+$ T cells (Man *et al*, ref 26; Man *et al*, ref 49), and there is every reason to think loss of IRF4 would have a similar effect here. Regarding the possibility of using an IRF4 mutant, please see our response to comment (8) of Reviewer #2.

- Figure 5 and S9: Overexpression of Irf4 seems to attenuate the boosting effect of Batf overexpression (Figure S9B, S10H, 5D). How does this fit to the proposed collaborative role between the two TF?

This single pairwise comparison between the effects of overexpressing BATF alone and overexpressing both BATF and IRF4 in original Figure S9D omits key information from the experiment. It is important to consider the totality of the experiment. First, overexpressing IRF4 alone leads to an improvement over controls at early time points (original Figures S9B-D), but does not result in survival of the mice past 30 days (Figure S9D). Combining overexpression of BATF with overexpression of IRF4 lengthens survival compared to overexpression of IRF4 alone (original Figure S9D). Overexpressing BATF in the context of endogenous levels of IRF4 (thus a higher BATF/IRF4 ratio) extends survival even further. These data are

consistent with functional cooperation between BATF and IRF4 in enhancing the tumor-killing ability of CAR TILs.

One simple explanation of the findindgs would be that IRF4 supports strong effector function through its binding at AICE sites, but that overexpressed IRF4 spills over to additional non-AICE binding sites where it is detrimental to CAR TIL function in the long term. Simultaneous overexpression of BATF might corral IRF4 to some extent at AICE sites, or even at low-affinity AICE sites that are irrelevant to CAR TIL function, but the corralling action could be less effective than with endogenous levels of IRF4.

- Moreover, the authors also conclude that Irf4 increases GranzymB expression but this increase is hard to be seen in in figure S9J, as there is no statistically significant difference in Figure S9J

We agree that there was no statistically significant difference in GzmB for the IRF4 and BATF+IRF4 cells in original Figure S9J [now Figure S7J]. We have corrected the text.

Minor points:

- Intro: It would be fair to also list the Immunity paper Utzschneyder et al. and Held, 2016 that initially reported on the role of the Tcf-1 population.

We cite the paper in the revised manuscript as ref 35.

- Figure 1H: shows a clear reduction in PD-1 expression. To judge the remaining expression level, it would be very informative to have a reference of the unstained background level.

We have added an isotype control to define the background level [Figure 1H].

- The gating strategy in figure 2 remains unclear. Do the plots show all cells or are they gated on Batf positive cells?

We gated on CD45 for lymphocytes, and utilized further markers to identify lymphocyte subsets, and Thy1.1 to identify CAR T cells. The gating strategy is shown in Figure S3, and we reference it in the legend to Figure 2.

- Figure 2: it remains unclear why the entire second figure is dedicated to illustrating mass-cytometry data which mainly reproduce what was already shown in figure 1. It is good data but could mostly be supplementary information.

Though we recognize that there could be different views on this, we still feel that the figure fits in the main text. However, we are open to presenting the figure as supplementary information, if the editors and reviewers find that option preferable.

- The authors state that KLRG-1 is upregulated following Batf transduction. The information is presently quite hidden in the presented datasets. To draw such a conclusion, a better representation of this finding should be presented.

Klrg1 is strongly upregulated only in a minor subset of cells, as was shown in original Figure S2D [now Figure S2G]. We have revised the sentence regarding KLRG-1 in the manuscript.

- There is a typo in line 340 “Figure S96B-D”.

We have corrected this.

- The illustration of the experimental system, in particular what “bZIP” could be better explained for less informed readers.

We now provide a clearer explanation of the experimental system in the legend to Figure S1.

Decision Letter, first revision:

Subject: Decision on Nature Immunology submission NI-A30443A

Message: Dear Anjana & Patrick,

Thank you for your response to the reviewers' comments on your manuscript "BATF and IRF4 cooperate to counter exhaustion in tumour-infiltrating CAR T cells". We are happy to inform you that if you revise your manuscript as indicated in your response rebuttal to the referees' comments and our editorial requirements your manuscript should be publishable in Nature Immunology.

Please revise your manuscript according with the reviewers' comments as outlined in your letter. At resubmission, please include a point-by-point response to the referees' comments, noting the pages and lines where the changes can be found in the revision. Please highlight the changes in the revised manuscript as well.

We are trying to improve the quality and transparency of methods and statistics reporting in our papers (please see our editorial in the May 2013 issue). Please update the Life Sciences Reporting Summary, and supplements if applicable, with any information relevant to any new experiments and upload it (as a Related Manuscript File) along with the files for your revision. If nothing in the checklist has changed, please upload the current version again.

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In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "BATF and IRF4 cooperate to counter exhaustion in tumour-infiltrating CAR T cells". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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We hope to receive your revised manuscript in 10 days, by 19th Apr 2021. Please let us know if circumstances will delay submission beyond this time. If you have any questions please do not hesitate to contact me.

Kind regards,

Laurie

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Reviewer #1 (Remarks to the Author):

The authors have thoroughly addressed earlier concerns with additional experiments, particularly ChIP-seq and RNA-seq of CAR T cells over expressing the mutant BATF-HKE protein enabling a comparison with their wild type BATF expressing counterparts. The accompanying bioinformatic analyses are consistent with the molecular mechanism involving BATF cooperativity with IRF4 and insightful in terms of genomic re-programming of CAR T cells before and after adoptive transfer, the latter in the TME.

Reviewer #2 (Remarks to the Author):

The work by Seo et al represents an important advance in the immunotherapy field. The authors have gone to some lengths to address our previous comments. While their response addresses some of my prior concerns, mainly in the form of commentary and response in the rebuttal letter, some outstanding issues remain.

In particular, given the competitive interactions of distinct bZip factors at DNA, it is dissappointing that the authors consider out of the scope of the present manuscript a mechanistic understanding of how BATF is actually working in the context of overexpression. While descriptive observations are presented, the paper lacks a clear model providing an understanding of which other bZip TFs BATF binding displaces, resulting in its effects on transcription and chromatin accessibility.

At this later stage in the description of BATF function in T cells (with many prior works in

this area) it is not unreasonable that molecular mechanisms by which its binding affects binding of other TFs at its binding sites are resolved. A variety of different assay systems could be used to assess this: for example, ChIP-PCR or ChIP-Seq-based measurements of the binding of other AP-1 factors such as c-Jun, JunD, Fos at BATF-bound loci in BATF-overexpressing cells would enable a clearer molecular model to be proposed. Alternatively, EMSA assays could be used to assess how BATF binding to DNA affects the binding of other transcription complexes. This remains an outstanding issue without which the manuscript only provides very superficial mechanistic detail. I hold out that this would be a helpful addition to an otherwise important manuscript.

Reviewer #3 (Remarks to the Author):

The authors have done an excellent job responding to comments and improving an already very strong manuscript. Overall, this is now a very data-intensive manuscript that combines a highly relevant observation with an extensive follow-up analysis.

I would have liked to see direct quantification of the number of Tcf1+ and Tcf1- cells after BATF overexpression. I tend to believe that BATF causes a selective increase in Tcf1- cells, but it would have been nice to see a quantification that the number of Tcf1+ cells remained constant.

Beyond that, I have only a couple of minor points:

The authors write in the point by point: "We added an isotype control to define the background level [Figure 1H]." I cannot see this control.

The authors write that Tox and Nr4as are "secondary transcription factors" that "cooperate with NFAT." I do not think there is sufficient evidence to classify exhaustion-associated TF in this way.

The authors clearly show that overexpression of BAFT increases anti-tumor activity, and they show that this is associated with increased numbers of tumor-specific cells as well as a significant degree of phenotypic change. It should be noted that it remains unclear which of the two effects is more important for the increased tumor control.

The authors write that "reprogramming" occurred. What is the evidence for reprogramming. Overexpression of BATF can alter cell differentiation from the beginning.

Author Rebuttal, first revision:

Reviewer #1

The authors have thoroughly addressed earlier concerns with additional experiments, particularly ChIP-seq and RNA-seq of CAR T cells over expressing the mutant BATF-HKE protein enabling a comparison with their wild type BATF expressing counterparts. The accompanying bioinformatic analyses are consistent with the molecular mechanism involving BATF cooperativity with IRF4 and insightful in terms of genomic re-programming of CAR T cells before and after adoptive transfer, the latter in the TME.

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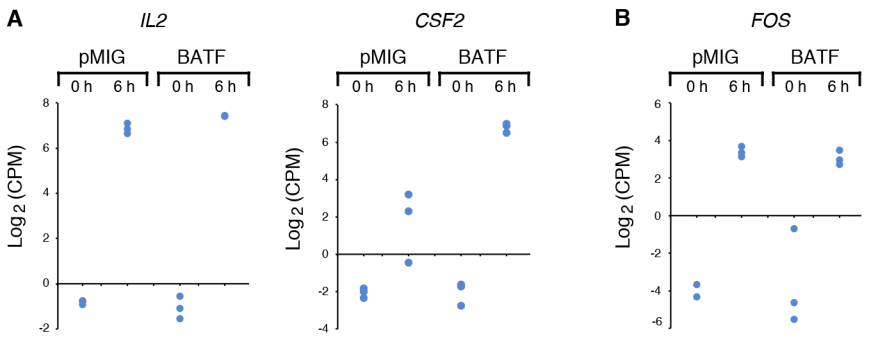
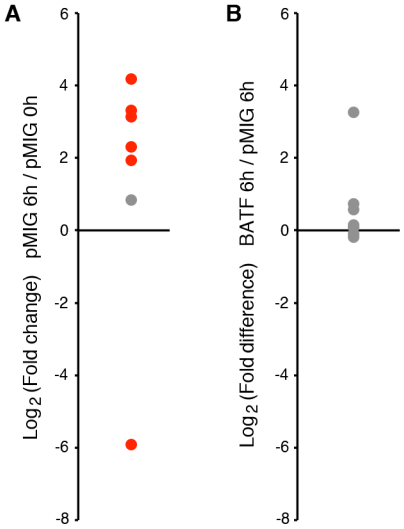
We accept the premise that it is past time to define BATF–AP1 competition rigorously in cases where it may occur. However, our ATAC-seq, ChIP-seq, and RNA-seq data did not point to competition as a principal mechanism for the enhanced effector function of CD8$^+$ T cells overexpressing BATF.

Historically, the idea that BATF (or its close homolog BATF3) competes with activating AP1 proteins or activating NFAT-AP1 complexes in T cells was first raised in two papers showing that BATF suppresses upregulation of an AP1 reporter in F9 teratocarcinoma cells and of a *CSF2* (*GM-CSF*) promoter reporter in human T cells [Echlin *et al*, 2000]; and that BATF3 suppresses upregulation of AP1, NFAT-AP1, and *IL2* promoter reporters in Jurkat cells [Iacobelli *et al*, 2000]. The reports documented that BATF-JUN complexes bind to AP1 sites in DNA, and showed by titrating BATF/BATF3 and FOS that BATF acts in competition with FOS to damp the transcriptional output, consistent with BATF-JUN heterodimers replacing FOS-JUN heterodimers in the relevant transcriptional complexes. The proposed action of BATF and BATF3 as negative modulators of AP1 transcriptional activity seemed consistent with the absence of a transcriptional activation domain in BATF and BATF3, although it required an unspecified alternative mechanism to account for genes



REVIEWER FIGURE 4 BATF overexpression and cytokine induction. **A**, Levels of *IL2* and *CSF2* mRNAs, from RNA-seq data, in resting cells (0h) and cells stimulated with αCD3/αCD28 (6h). **B**, Levels of *FOS* mRNA in the same experiments.

upregulated by the BATF proteins. Some years later, that alternative mechanism was supplied, with the evidence that BATF and BATF3 recruit IRF4 (or IRF8) to activate transcription of many target genes in dendritic cells, Th2 cells, and Th17 cells [Li *et al*, 2012; Glasmacher *et al*, 2012; Tussiwand *et al*, 2012; reviewed in Murphy *et al*, 2013]. The latter findings led, perhaps, to a diminished experimental emphasis on the proposed negative modulatory role. An important recent paper [Lynn *et al*, 2019] has focused attention once again on functional competition between BATF and JUN, in CD8⁺ T cells that express a tonically signalling chimeric antigen receptor (CAR) and overexpress JUN. In this case, the proposed mechanism is that an elevated ratio of cellular JUN to cellular BATF causes displacement of AP1-IRF complexes from sites that drive expression of key exhaustion-related genes.



REVIEWER FIGURE 5 Data for selected genes reported as downregulated in JUN-expressing cells. The genes common to our work (CD8⁺ T cells) and Lynn *et al* (CD4⁺ and CD8⁺ T cells) are *BATF3*, *GZMB*, *LAG3, LTA*, *TNFRSF9*, *JUNB*, and *ENTPD1*; *IL13* was not expressed in CD8⁺ T cells. *Red symbols*, significant differences, $|Log_2(\text{Fold change})| > 1$, adj $p < 0.05$; *grey symbols*, NS. **A**, Expression of six of the genes is significantly altered by αCD3/αCD28 stimulation. **B**, None of the genes is differentially upregulated in BATF-overexpressing cells compared to pMIG control cells. A single gene, *ENTPD1*, stands out for higher average expression in BATF-overexpressing cells, but the difference is not statistically significant (adj $p = 0.19$).

Invoking BATF–AP1 competition to explain our findings faces a conceptual difficulty at the outset: Competition between BATF and activating AP1 proteins does not explain in a straightforward way why overexpression of BATF has a similar effect to overexpression of JUN, rendering CD8⁺ TILs more capable of controlling a tumor. The hypothesis is also contradicted by the data. First, there was no obvious competition with FOS for cytokine induction using the measures reported in Iacobelli *et al* and in Echlin *et al*. Overexpression of BATF under our conditions did not depress *IL2* induction in response to αCD3/αCD28 stimulation, and strikingly elevated *CSF2* induction [**REVIEWER FIGURE 4A**]. The failure of BATF overexpression to suppress these cytokines was not due to a compensating increase in FOS expression, since there was no significant difference in *FOS* induction between BATF-overexpressing cells and control cells [**REVIEWER FIGURE 4B**]. Second, our data did not lend support to a mechanism where the BATF:JUN ratio determines expression of exhaustion-related genes. The prediction, if there were such functional competition, is that overexpressing BATF would have effects on transcription opposite to those seen in JUN-overexpressing cells, particularly for the genes whose expression is decreased in JUN-overexpressing cells. This was not the case for the seven JUN-downregulated genes listed in Lynn *et al*, Extended Data Fig. 6b, that are expressed in CD8⁺ T cells. Five of these genes were significantly induced and one was downregulated in pMIG control cells upon αCD3/αCD28 stimulation [**REVIEWER FIGURE 5A**], verifying that they are sensitively attuned to cellular signalling in our conditions, but none showed differential expression when comparing BATF-overexpressing cells with pMIG control cells [**REVIEWER FIGURE 5B**]. (A further ~160 genes that were not named explicitly in the Lynn *et al* paper were reported to be differentially downregulated in JUN-overexpressing cells. However, it seems clear already that the general prediction regarding BATF

overexpression and JUN overexpression is not supported, without the need to download all the RNA-seq data for JUN-overexpressing cells from the GEO database and re-analyze it.)

Thus, while BATF may compete with activating AP1 proteins under some conditions, BATF-overexpressing CD8⁺ T cells are not a promising case for investigating this competition mechanism. We would be happy to add a paragraph to the Discussion elaborating on this point.

Literature cited—

Echlin DR, Tae H-J, Mitin N, Taparowsky EJ (2000) B-ATF functions as a negative regulator of AP-1 mediated transcription and blocks cellular transformation by Ras and Fos. *Oncogene* 19, 1752-1763.

Glasmacher E, *et al* (2012) A genomic regulatory element that directs assembly and function of immune-specific AP-1–IRF complexes. *Science* 338, 975-980.

Iacobelli M, Wachsman W, McGuire KL (2000) Repression of IL-2 promoter activity by the novel basic leucine zipper p21^{SNFT} protein. *J Immunol* 165, 860-868.

Li P, *et al* (2012) BATF-JUN is critical for IRF4-mediated transcription in T cells. *Nature* 490, 543-546.

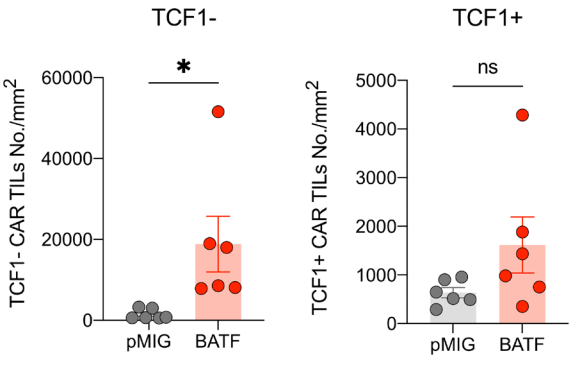
Lynn RC, *et al* (2019) c-Jun overexpression in CAR T cells induces exhaustion resistance. *Nature* 576, 293-300.

Murphy TM, Tussiwand R, Murphy KM (2013) Specificity through cooperation: BATF-IRF interactions control immune regulatory networks. *Nat Rev Immunol* 13, 499-509.

Tussiwand R, *et al* (2012) Compensatory dendritic cell development mediated by BATF–IRF interactions. *Nature* 490, 502-507.

Reviewer #3

The authors have done an excellent job responding to comments and improving an already very strong manuscript. Overall, this is now a very data-intensive manuscript that combines a highly relevant observation with an extensive follow-up analysis.



REVIEWER FIGURE 6

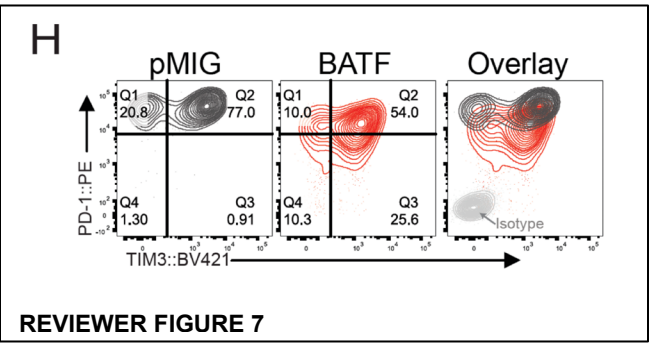
I would have liked to see direct quantification of the number of Tcf1+ and Tcf1- cells after BATF overexpression. I tend to believe that BATF causes a selective increase in Tcf1- cells, but it would have been nice to see a quantification that the number of Tcf1+ cells remained constant.

The data are shown in **REVIEWER FIGURE 6**. We have added this information to the manuscript as Figure 1k.

Beyond that, I have only a couple of minor points:

The authors write in the point by point: "We added an isotype control to define the background level [Figure 1H]." I cannot see this control.

The isotype control was included in the original Figure panel 1h, but was drawn in too light a shade of grey. We now use a darker shade of grey in Figure 1h, as shown for reference in **REVIEWER FIGURE 7**.



REVIEWER FIGURE 7

The authors write that Tox and Nr4as are "secondary transcription factors" that "cooperate with NFAT." I do not think there is sufficient evidence to classify exhaustion-associated TF in this way.

The wording was intended to convey only that TOX and NR4A proteins are transcriptional targets of NFAT and, like NFAT, act to promote the exhaustion transcriptional program. We have rephrased the sentence, which now appears in Supplementary Notes, p. 1, lines 5-6.

The authors clearly show that overexpression of BATF increases anti-tumor activity, and they show that this is associated with increased numbers of tumor-specific cells as well as a significant degree of phenotypic change. It should be noted that it remains unclear which of the two effects is more important for the increased tumor control.

Agreed. We have included a statement in Discussion that both increased CD8$^+$ TIL numbers and altered TIL phenotypes are likely to contribute to enhanced tumor control (p. 8, lines 401-402).

The authors write that "reprogramming" occurred. What is the evidence for reprogramming. Overexpression of BATF can alter cell differentiation from the beginning.

We have avoided the term ‘reprogramming’ in the revised text. In four instances, it has been replaced with terms that do not carry any specific connotations regarding cell differentiation (p. 3, line 118; p. 4, line 159; p. 5, line 226; p. 8, line 391), and in one instance the phrase with ‘reprogrammed’ has been omitted entirely (p. 3, lines 132-133).

Decision Letter, second revision:

Subject: Your manuscript, NI-A30443B

Message: Our ref: NI-A30443B

3rd May 2021

Dear Dr. Rao,

Thank you for your patience as we’ve prepared the guidelines for final submission of your Nature Immunology manuscript, "BATF and IRF4 cooperate to counter exhaustion in tumour-infiltrating CAR T cells" (NI-A30443B). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if

you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments and please make sure to upload your checklist.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Immunology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "BATF and IRF4 cooperate to counter exhaustion in tumour-infiltrating CAR T cells". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Best regards,

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Phone: 212 726 9207
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E-mail: immunology@us.nature.com

On behalf of

Laurie A. Dempsey, Ph.D.
Senior Editor

nature research

Nature Immunology
l.dempsey@us.nature.com
ORCID: 0000-0002-3304-796X

Final Decision Letter:

Subject: Decision on Nature Immunology submission NI-A30443C

Message: In reply please quote: NI-A30443C

Dear Anjana & Patrick,

I am delighted to accept your manuscript entitled "BATF and IRF4 cooperate to counter exhaustion in tumor-infiltrating CAR T cells" for publication in an upcoming issue of Nature Immunology.

The manuscript will now be copy-edited and prepared for the printer. Please check your calendar: if you will be unavailable to check the galley for some portion of the next month, we need the contact information of whom will be making corrections in your stead. When you receive your galleys, please examine them carefully to ensure that we have not inadvertently altered the sense of your text.

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Kind regards,

Laurie

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