EMBO
reports

Suppression of Ca^{2+} Signals by EGR4 controls Th1 differentiation and anti-cancer immunity *in vivo*

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1st Editorial Decision 22 July 2019

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript, the referee reports from The EMBO Journal (attached below), and your 'preliminary point-by-point response'. All referees acknowledge the potential interest of the findings. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn.

EMBO reports emphasizes novel functional over detailed mechanistic insight, but asks for strong in vivo relevance of the findings, and clear experimental support of the major conclusions. Thus, we will not require to address points regarding more refined mechanistic details. In that light, it will be necessary that in a revised manuscript you address experimentally points questioning the main conclusions of the study, and all technical concerns, or points regarding the experimental designs, model systems used, or data presentation. I think it will be important to also address the question if the observed phenotype is T cell-intrinsic, and to provide the time course experiment (referee #1, point 3). However, we would not require the RNA-seq. analysis (referee #2, point 4). Taken together, the revision as suggested by your preliminary p-b-p-response seems very reasonable.

Given the constructive referee comments, we would like to invite you to revise your manuscript for EMBO reports with the understanding that the referee concerns must be addressed in the revised manuscript (as indicated above) and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review (using the same referees that have assessed the study before). It is our policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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for the revisions so that we can discuss the revisions further.

When submitting your revised manuscript, please also carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision. When submitting your revised manuscript, we will require:

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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Moreover, I have these editorial requests:

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

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

REFEREE REPORTS

Referee #1:

The manuscript entitled "Suppression of Calcium Signals by EGR4 Controls Th1 Differentiation and Anti-cancer Immunity In Vivo" by Mookerjee-Basu et. al. describes studies undertaken to understand the role of the transcription factor EGR4 in T cell differentiation and function. Little is known about EGR4 in lymphocytes so this represents a novel set of studies with significant impact. The authors argue that EGR4 deficiency leads to a Th1 bias primarily due to calcium/NFAT induced production of IFN gamma. The manuscript describes a negative regulatory role for EGR4 that is evident from the hyperactivated phenotype of EGR4 deficient T cells. The authors attribute hyperactivation, including a significant de-repression of IFN gamma production, to the induction of calcium-activated potassium channels. They propose that membrane hyperpolarization due to calcium activation of KCa3.1 channels promotes higher amplitude and/or persistent increases in cytoplasmic calcium and that this results in NFAT hyper-activation and elevated expression of IFN gamma in EGR4 deficient cells. They also suggest that EGR4 deficient mice have enhanced anti-tumor immunity, because of defects in Treg generation and T cell exhaustion. The mechanistic model proposed is comprehensive and complex, and while the overall impact of EGR4 deficiency

on enhanced anti-tumor immunity is solid, some of the underlying mechanistic studies are less convincing in present form.

Major Concerns:
Figure 1 shows that EGR4 mRNA is expressed transiently in WT T cells, with a peak at 2 hours and decay to resting levels by 20 hours. These studies only assess mRNA and NOT protein. At least for EGR4, parallel protein analysis should be shown. The authors also allude to a statistically significant increase in CD4 and CD8 T cells in EGR4 KO mice, but no indication of level of significance is shown on Figure 1E. In Figure 1G, it appears as if the reported difference in CM cells (among CD8+ T cells) is a comparison to EGR1 KO cells but NO difference is shown from WT cells.

A general concern with the FACS analysis in this manuscript is the absence of treatment controls, genotype controls, and staining controls (isotype or FMO controls). In many instances, the basis for gating is not clear and not convincing. For example, the authors must show side by side comparisons of Tregs (CD25 vs. FoxP3) for different genotypes and controls and the purported subsets. There are no controls for 1I and 1J. In some instances, different gates were applied to WT and EGR4 KO cells. The authors need to include in the supplement representative FACs plots that illustrate the gating strategy for each population and genotype to substantiate the subset analysis shown in Figure 1 and other figures.

In Figure 2, the authors should provide a timecourse of EGR4 expression in WT CD4 and CD8 T cells using "weak" and "strong" stimuli as a foundation for subsequent conclusions about the relationship between stimulus strength and EGR4 dependent responses of the cells. The authors state "The ability of weak versus strong TCR signals to induce different levels of EGR and consequently different cellular responses is well-established." But this needs to be calibrated by showing this is also true for their conditions. A full timecourse is an essential.
Another perplexing point that is not adequately addressed is the fact that resting EGR4 -/- cells make several cytokines. This suggests that "naïve" EGR4 -/- cells are "activated" by antigen. The implications of this pre-activation seem to be overlooked and could impact any interpretation of findings. This seems to be particularly relevant to calcium measurements, which are never measured in freshly isolate populations under conditions of TCRbeta or CD3/CD28 stimulation. It is essential to know if activated naïve cells exhibit an initial calcium phenotype. This is different than showing that calcium levels in WT and KO resting unstimulated cells are not different. Indeed, based upon the authors results one would expect that the initial TCR induced changes in calcium over an initial period of 15-30 minutes are different in freshly isolated WT and EGR4 -/- cells.

Another confusing result is the cytokine bead assays after 48 hours of culture without stimulus in EGR4-/- cells, and then the impact of stimulation on the levels of the same cytokines. This suggests that EGR4-/- cells are "activated". Again, as with calcium, what are the implications of this in terms of a mechanistic hypothesis?

In the text describing Figure 3 on p.7 the authors conclude, "Importantly, the role of EGR4 in control of cytokine gene expression is non-redundant, i.e. distinct from other EGR family members, as it cannot be compensated for by EGR1." This conclusion is not well founded as the authors ONLY compared the response to EGR1 KO cells. Consequently, the language should be tempered. Similar broad statements are made elsewhere in the manuscript and these should likewise be scaled back. Also, the data in Figure 3L are not substantiated nor necessarily significant, and the following conclusion should be removed (or multiple tests with statistical analysis presented to establish the accuracy of this conclusion): "Similar polarization of CD8+ T cells with IL-2 and IL-12, which is known to promote Tc1 CD8+ fate (Joshi et al, 2007; Kalia et al, 2010; Pipkin et al, 2010), also induced greater Granzyme B and Perforin induction in the absence of EGR4 than in WT cells (Fig. 3L). Furthermore, in the next paragraph the authors refer to data not shown, this needs to be shown or the comment removed. Hyper-induction of TNFα in EGR4-deficient CD4+ T cells under Treg conditions (data not shown) ...

In CD8+ T cells, increased NFAT is observed at 2 hours and in CD4+ T cells at 8 hours. Based upon these data, the authors conclude that EGR4 acts to limit the "strength and duration" of NFAT activation, inferring a difference in calcium signaling. The mechanistic prediction of this result is that calcium levels would be higher at times when NFAT localization to the nucleus is greater. Indeed, the average cytoplasmic calcium concentration is higher at 2 hours in EGR4 KO than WT

CD4+ T cells, and this correlates with greater levels of nuclear NFAT. However, this is a very unusual analysis first because these 3 minutes snapshots provide a very limited and incomplete snapshot of calcium dynamics and a critical control is missing. An underlying prediction of this approach is that antigen/TCR induced calcium signals are regulated differently in the absence of EGR4, and that this reflects a change in the K channel regulated membrane potential. However, as unstimulated EGR4 KO T cells produce IFN gamma in the absence of stimulation, it is also likely that the starting states of WT and EGR4 KO T cells are already different. Yet, the authors do not look at TCR induced calcium signals in resting cells but only levels in unstimulated cells. This is particularly important to address because the authors demonstrate (Figure S3) that KCa3.1 and Kv1.3 expression are elevated in resting cells. One would expect initial TCR/CD3 induced calcium signals to be significantly different in resting cells. Furthermore, this is not evident in the initial induction of nuclear NFAT. This is unexpected and needs to be addressed. The fact that these channels are elevated in resting cells complicates any mechanistic interpretation of results. Indeed, KCa3/1 and Kv1.3 expression are normally induced following TCR activation of T cells, so these resting cells are more like activated T cells. It would be helpful if the authors performed a timecourse analysis of Kv1.3, PMCA4a, and KCa3.1 expression to better explain the calcium signals.

It is also strange that in the EGR1 -/- cells there happens to be nearly synchronized responses at both 2 and 20 hours. This suggest there may be some artefact to these measurements, possibly a UV illumination induced change. A necessary control for these experiments both to calibrate results to known published responses and to assess the possibility that changes observed are already baked into unstimulated cells is to perform measurements of the initial calcium signals induced in real time by TCR and CD3/CD28 in unstimulated freshly isolated cells over 15-30 minutes. The authors should then analyze differences or similarities. This would also speak to any possible pre-existing role for KCa channels in resting cells. Also, if EGR4 normally suppresses KCa expression why do WT cells exhibit a progressive increase in "steady state" levels and EGR4 -/- exhibit a transient increase and then decrease at 20 hours, while at 20 hours the levels of nuclear NFAT in WT and DKO are the same? It seems as if the interpretations here reflect expectations rather than a purely objective read of the data.

I am also confused by the EGR1 -/- results and wonder what the basis is for muted signals (2 and 20 hours). Would a further analysis of this inform a general mechanism of control over calcium by the EGRs? The authors never discuss the implications of this apparent inhibition of calcium signaling in EGR1 -/- cells. The authors should also confirm that BPT2 blocks NFAT localization in both WT and EGR4-/- cells to close the circle on this mechanism. Furthermore, although calcium levels are higher in EGR4 -/- cells, the delayed effect of BPT2 inhibition is perplexing and suggest that something else is at play here. Why does BPT2 not equally effect IL-2 production, which is a classic cytokine whose expression is exquisitely sensitive to calcium/NFAT. Why doesn't BPT2 fully suppress IL-2, not just the "hyperinduction". Why is a significant portion of IL-2 production in EGR4-/- cells calcium insensitive? Finally, as the goal of this experiment was to demonstrate cause and effect between EGR4 expression, calcium levels, and NFAT and differences are attributed to K channels, the authors should evaluate the impact of this blocker and the K channel inhibitors on NFAT localization.

Given the established role for Kv1.3 in IL-2 production by and proliferation of T cells, why did the authors NOT test the effect Shk-Dap22 on calcium or proliferation to validate these mechanisms in WT cells and establish a basis for comparing a role in EGR4 -/- cells. At the very least these data would be an excellent positive control and Shk-Dap22's impact on WT and KO cells would more completely inform the mechanism of calcium regulation and its consequences. Indeed, the authors do NOT establish that elevated Kv1.3 is NOT involved in the increased proliferation of EGR4-/- cells.

In general, the differences in tumor growth and metastasis in WT and EGR4-/- mice are intriguing and significant. The phenotype is convincing and well documented. However, the mechanistic analysis is less compelling. This reflects a general concern with this manuscript, that almost none of the FACS analysis in these studies is properly controlled and the gating strategies, and in some cases the gating is not validated or properly performed. This applies to all analysis of Tregs and FACS plots from which summarized analyses are shown in figures. For each subset, examples of primary FACS plots including the subset gating and controls used to establishing gates/regions should be

included in the supplement. This is especially a concern for data in Figures 9G, H, I, and J (and this raises questions about the analysis in 9B) and in Figures 10 D-K, which also has an additional problem in that the gates are not identical for different genotypes. Additional concerns revolve around the analyses of FR4 and any conclusions drawn from these results. Without proper controls and explanation of a gating strategy, the FR4 analysis is incomprehensible. Consequently, conclusions about anergy based upon this analysis are weak. Furthemore, if anergy is really a factor, this should be evaluated in functional assays. Another conclusion that EGR4-/- cells have increased killing capacity could be tested directly and would strengthen this mechanism.

Given these concerns, until these issues are addressed, the results do NOT support the mechanistic conclusions that the anti-tumor phenotype reflects a role for Tregs or exhaustion. Consequently, the experimental basis of the main conclusion of this section (Thus, loss of EGR4 not only attenuates induction of anergy/exhaustion in the immunosuppressive tumor microenvironment, but also promotes cytotoxic effector differentiation) needs to be better established

In summary, while this is an important topic and clearly EGR4 plays an important role in T cell activation and function, this manuscript is not acceptable in its current form. However, if the concerns outlined are addressed, it would be suitable for publication.

Referee #2:

Egr1, 2, and 3 are recognised for their roles in the development and peripheral functions of T and NKT cells. However, the function of Egr4 in T cells is still unknown. The authors analysed T cells from Egr4 KO mice and found increased Th1 and effector function of peripheral T cells, alongside decreased Treg. Although this function of Egr4 in T cells is novel, the paper suffers from some major problems, as indicated below.

Major points

1. Explanation of results obtained a few days after stimulation, despite Egr4 expression being both very transient, and at a very low level. It is consistent with previous findings; the author has demonstrated a very transient and low level expression at mRNA levels in T cells following anti-CD3 stimulation in vitro. However, the majority of results obtained in vitro represent only a few days post-stimulation. If there is indeed the indirect effect of Egr4, authors must demonstrate the rescue effect of Egr4 by overexpression Egr4 into wild type or Egr4 KO T cells.
2. Lack of support for the Hyper-proliferation conclusion. Aside from TCRb stimulation, the proliferation results are less convincing. This may be due to the high doses of anti-CD3. A dose-dependent result and cell cycle analysis will give better results if Egr4-/- T cells are hyper-proliferative in response to TCR and costimulatory stimulation.
3. Lack of mechanistic explanation for the over production of IFNg by CD4, and the increased effector function of CD8. Increased IFNg production and CD8 effector function have been reported from Egr2/3-/- T cells and these have resulted from the defect expression of repressors, uncontrolled T-bet activity and/or Lag3 function. Authors must assess the TFs defining Th1 and CD8 functions, as per stat5 and T-bet.
4. RNAseq results are missing. TF function is translated by target genes, or genes that have been regulated indirectly.
5. Explanation of the cause of TCR signalling assays. Authors showed that Egr4 mRNA are not expressed until after anti-CD3 stimulation for hour or two, then rapidly retracted to background levels.However, TCR signalling is much more rapid than the induction of Egr4. How can we explain this?
6. Lack of mechanisms for supporting Treg. Although Foxp3+CD4 T cells are reduced, the development of Treg and their function in suppressing effector T cells are not examined.
7. B16 is a melanoma model. We do not understand why B16 is used to investigate tumour metastasis. TIL are highly inflammatory, but the number of cases does not increase. Authors must examine proliferation markers, as terminally differentiated T cells are highly apoptotic.
8. Excessive production of IFNg in tumour microenvironments is associated with increased metastasis of the tumour. Authors should examine the tumour specificity of TIL by in vitro stimulation with tumour cells, or DCs loaded with tumour lysates. The hyper-inflammatory results from both TIL and T cells in spleens may indicate aresponse that is innate.

9. The gating between the control and KO is not consistent in Fig. 9G, H, I, J and Fig. 10. J, K.

Referee #3:

How some EGR proteins, such as EGR1 or EGR2, regulate different aspect of peripheral T cell function has been extensively studied. However whether EGR4 does also participate in the regulation of T cell responses has not been determined yet. This manuscript presents a comprehensive characterization of the role that EGR4 plays in T cells and, using a genetic model where that gene has been knocked out, identifies novel non-redundant roles of EGR4 in T cell differentiation. T cells form Egr4-/- mice show increased production of some effector cytokines that leads to enhanced Th1 type responses with reduced Treg differentiation. Consequently, mice that are deficient in EGR4 show enhanced capacity to control the development of B16 melanoma metastasis.

Whereas the data is very exciting and the results clearly point to a novel role for EGR4 in T cell differentiation, there many questions that remained not fully answered regarding the identification of the targets of EGR4 and of the mechanisms through which this TF regulates them.

In that sense, are the EGR4 effects on calcium alone responsible for the increased in proliferation and cytokine expression? Is that is the case why is there only minimal effects on IL-2 expression compared to other cytokines (Fig. A-F) when IL-2 transcription is clearly dependent on calcium signaling? Are the frequency and amplitude of the modulation of calcium currents preferentially induce specific cytokines? If so how? Does EGR4 directly or indirectly repress the transcription of any of those genes?

The increase in calcium entry is proposed to be likely mediated by the increased expression of two potassium channels. The experiments performed to prove this point are carried out using channel inhibitors. One would expect that no matter if the expression of those channels is elevated or not in Egr4-/- cells, blocking their function would have a inhibitory effect on calcium currents in any T cell. It would have been a much better approach, first to confirm that K currents are affected and then to use siRNA to silence the expression of those channels to levels as similar as possible to the ones found in wt cells.
Reduced Treg differentiation is attributed to either an effect of the loss of EGR4 on Helios expression or to the consequences of increased expression of TNF in Egr4-/- T cells. However, none of these hypotheses are tested. Does blocking TNF restore Treg differentiation? Is Helios expression dependent on Egr4 (ChIP, reporter experiments...) or is it a secondary effect?

The characterization of Th differentiation innEgr4-/- cells shows increased IFNg expression under all conditions w/o major differences in the production of other lineage defining cytokines. However, this data is presented only as secretion measured by ELISA, which prevents determining whether a pool of cells remains undifferentiated and expressing high levels of IFNg or if the absence of Egr4 leads to the generation of abnormally differentiated cells that express both IFNg and other cytokines. Intracellular staining for those cytokines would be necessary to answer this question.

The data from the tumor model is compelling but the model used is a full body KO and cannot conclusively prove that the better control of metastases seen in Egr4-/- mice responds to the development of increased Th1 like responses and/or to decreased Treg differentiation. In order to do so, in the absence of a floxed mouse, more specific approaches should be followed, such as BM chimeras spiking TCR-/- BM with EGR4-/- BM, or at least adoptive transfers of tumor antigen specific Egr4-/- T cells (e.g. TRP-1 system for B16) or even using surrogate antigens. It is also not clear why the i.v. injection model was used, which delivers a very high load of cells with metastatic potential, instead of a more physiologic model (e.g. B16F10) that would also allow evaluation of primary tumor control. There are also some concerns with the different results obtained with the IVIS system and the actual count of metastases. Are those discrepancies due to increased number but reduced size of lung metastases in the Egr4-/- mouse or are they just due to some technical limitation of the IVIS system?

The authors keep referring to an role of Egr4 on thresholds of activation, and based those observations in the comparison of anti-CD3 or with anti-CD3 and anti-CD28 antibodies. Strictly

speaking, these conditions do not represent different strengths of activation but rather the presence or absence of costimulation, and may support that effects seen may or may not be costimulation dependent. Analyses using different strengths of TCR stimuli would be needed to determine if Egr4 may control thresholds for activation.

Minor points:
FACS gating is not offered in some analysis (e.g. Fig. 1). To better evaluate the characteristics of all cell populations analyzed, sample flow graphs with gating strategy should be provided at least as Suppl. Data.

Although it might be due to the conversion to pdf, the quality of the IF in Fig4 is quite low. In any case the differences in NFAT nuclear translocation, though statistically significant, are low in magnitude. Are they biological relevant? Can they explain the marked changes in cytokine expression and activation-induced proliferation seen in Egr4-/- T cells?

Modulation of EGR1-3 expression in WT cells as shown in Fig. 1 is obscured by the huge compensatory increases that occur in EGR deficient mice. As one of the important points made by the authors to explain non-redundant functions of EGR4 and other EGR protein, is that their kinetics of expression are very different (biphasic vs sustained), it would make it easier for the reader to appreciate those differences if graphs showing only the regulation of Egr gene expression in wt cells was also offered.

The authors claim that "However, there was a slight but statistically significant increase in naïve CD4+ and CD8+ T subsets in EGR4-/- mice (Fig.1E)" However, there are no such stats shown for naive CD8+ T cells. Furthermore, the reference should be to Fig 1F-G.

Should Fig 1K be a dot plot?

1st Revision - authors' response 8 January 2020

Referee #1:

Major Concerns:

1) Figure 1 shows that EGR4 mRNA is expressed transiently in WT T cells, with a peak at 2 hours and decay to resting levels by 20 hours. These studies only assess mRNA and NOT protein. At least for EGR4, parallel protein analysis should be shown. The authors also allude to a statistically significant increase in CD4 and CD8 T cells in EGR4 KO mice, but no indication of level of significance is shown on Figure 1E. In Figure 1G, it appears as if the reported difference in CM cells (among CD8+ T cells) is a comparison to EGR1 KO cells but NO difference is shown from WT cells.
We agree that protein analysis would have been desirable and have expended considerable effort in this regard. Unfortunately, we determined that there is no commercially available antibody that can convincingly detect EGR4 expression by Western analysis. We are actively working towards the design of our own antibody. However, at present we are forced to rely on mRNA detection by qPCR.
Regarding figure 1, our apologies for errors in our description. There are no statistically significant differences in CD4 and CD8 T cells numbers in EGR4KO mice. Further, the reviewer was correct regarding the effect on CM cells. We have revised the text accordingly.

2) A general concern with the FACS analysis in this manuscript is the absence of treatment controls, genotype controls, and staining controls (isotype or FMO controls). In many instances, the basis for gating is not clear and not convincing. For example, the authors must show side by side comparisons of Tregs (CD25 vs. FoxP3) for different genotypes and controls and the purported subsets. There are no controls for 1I and 1J. In some instances, different gates were applied to WT and EGR4 KO cells. The authors need to include in the supplement representative FACs plots that illustrate the gating strategy for each population and genotype to substantiate the subset analysis shown in Figure 1 and other figures.

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We apologize for deficiencies in the presentation of our data in the first submission. We have gone through our data with considerable care to ensure that gating strategies are clear and matched for all data. Gating strategies are included in the supplementary information.

3) In Figure 2, the authors should provide a timecourse of EGR4 expression in WT CD4 and CD8 T cells using "weak" and "strong" stimuli as a foundation for subsequent conclusions about the relationship between stimulus strength and EGR4 dependent responses of the cells. The authors state "The ability of weak versus strong TCR signals to induce different levels of EGR and consequently different cellular responses is well-established." But this needs to be calibrated by showing this is also true for their conditions. A full timecourse is an essential.
We completely agree with the reviewer and have provided a complete timecourse in revised figure 2, showing that EGR4-/- T cells are hyperproliferative in response to both weak and strong TCR signals.

4) Another perplexing point that is not adequately addressed is the fact that resting EGR4 -/- cells make several cytokines. This suggests that "naïve" EGR4 -/- cells are "activated" by antigen. The implications of this pre-activation seem to be overlooked and could impact any interpretation of findings. This seems to be particularly relevant to calcium measurements, which are never measured in freshly isolate populations under conditions of TCRbeta or CD3/CD28 stimulation. It is essential to know if activated naïve cells exhibit an initial calcium phenotype. This is different than showing that calcium levels in WT and KO resting unstimulated cells are not different. Indeed, based upon the authors results one would expect that the initial TCR induced changes in calcium over an initial period of 15-30 minutes are different in freshly isolated WT and EGR4 -/- cells.
We recognize the reviewer's intriguing hypothesis that EGR4-/- cells may not actually be naïve. However, we have analyzed the initial TCR-induced Ca^{2+} response, observing no difference between WT and EGR4-/- cells (Figure 5A-C). Since EGR4-/- T cells do not exhibit resting Ca^{2+} oscillations or exhibit increased sensitivity to TCR signals (before or after crosslinking), we have no evidence to support the possibility that EGR4-/- T cells are pre-activated.

5) Another confusing result is the cytokine bead assays after 48 hours of culture without stimulus in EGR4-/- cells, and then the impact of stimulation on the levels of the same cytokines. This suggests that EGR4-/- cells are "activated". Again, as with calcium, what are the implications of this in terms of a mechanistic hypothesis?
We fully understand the reviewer's comment that EGR4-/- T cells appear to show an "activated" phenotype with respect to cytokine expression. However, it's important to note that these cells do not appear activated according to other criteria, in particular surface marker expression profile. Accordingly, for studies examining cytokine expression and CFSE staining, we explicitly sorted cells with a non-activated CD44- phenotype for these studies (and confirmed by FACS that these cells were also CD69, $CD62l^{hi}$). The manuscript has been modified to clarify this point. As such, while we appreciate the attractiveness of the reviewer's hypothesis, we have no basis to support it. We might speculate that some gene expression changes induced by TCR stimulation at earlier time points (e.g. during thymic development) can become permanent in the absence of EGR4, i.e. that EGR4 normally plays a role in reversing some of these events. In the revised manuscript, we have expanded our discussion of this issue.

6) In the text describing Figure 3 on p.7 the authors conclude, "Importantly, the role of EGR4 in control of cytokine gene expression is non-redundant, i.e. distinct from other EGR family members, as it cannot be compensated for by EGR1." This conclusion is not well founded as the authors ONLY compared the response to EGR1 KO cells. Consequently, the language should be tempered. Similar broad statements are made elsewhere in the manuscript and these should likewise be scaled back. Also, the data in Figure 3L are not substantiated nor necessarily significant, and the following conclusion should be removed (or multiple tests with statistical analysis presented to establish the accuracy of this conclusion): "Similar polarization of CD8+ T cells with IL-2 and IL-12, which is known to promote Tc1 CD8+ fate (Joshi et al, 2007; Kalia et al, 2010; Pipkin et al, 2010), also induced greater Granzyme B and Perforin induction in the absence of EGR4 than in WT cells (Fig. 3L). Furthermore, in the next paragraph the authors refer to data not shown, this needs to be shown or the comment

removed. Hyper-induction of TNFα in EGR4-deficient CD4+ T cells under Treg conditions (data not shown) ...
We understand and agree with the reviewer that more care was needed in our language. The experiment depicted in original figure 3L (Figure S4 in the revised manuscript) was repeated 2 times with similar results, as is indicated in the legend. All references to “data not shown” have been deleted.

7) In CD8+ T cells, increased NFAT is observed at 2 hours and in CD4+ T cells at 8 hours. Based upon these data, the authors conclude that EGR4 acts to limit the "strength and duration" of NFAT activation, inferring a difference in calcium signaling. The mechanistic prediction of this result is that calcium levels would be higher at times when NFAT localization to the nucleus is greater. Indeed, the average cytoplasmic calcium concentration is higher at 2 hours in EGR4 KO than WT CD4+ T cells, and this correlates with greater levels of nuclear NFAT. However, this is a very unusual analysis first because these 3 minutes snapshots provide a very limited and incomplete snapshot of calcium dynamics and a critical control is missing. An underlying prediction of this approach is that antigen/TCR induced calcium signals are regulated differently in the absence of EGR4, and that this reflects a change in the K channel regulated membrane potential. However, as unstimulated EGR4 KO T cells produce IFN gamma in the absence of stimulation, it is also likely that the starting states of WT and EGR4 KO T cells are already different. Yet, the authors do not look at TCR induced calcium signals in resting cells but only levels in unstimulated cells. This is particularly important to address because the authors demonstrate (Figure S3) that KCa3.1 and Kv1.3 expression are elevated in resting cells. One would expect initial TCR/CD3 induced calcium signals to be significantly different in resting cells. Furthermore, this is not evident in the initial induction of nuclear NFAT. This is unexpected and needs to be addressed. The fact that these channels are elevated in resting cells complicates any mechanistic interpretation of results. Indeed, KCa3/1 and Kv1.3 expression are normally induced following TCR activation of T cells, so these resting cells are more like activated T cells. It would be helpful if the authors performed a timecourse analysis of Kv1.3, PMCA4a, and KCa3.1 expression to better explain the calcium signals.
We appreciate the reviewer’s insightful comments, which provide an interesting direction for future studies. However, as the reviewer states, the proposed studies would assist in “mechanistic interpretation” of our results, and as such are beyond the scope for the current submission.

8) It is also strange that in the EGR1 -/- cells there happens to be nearly synchronized responses at both 2 and 20 hours. This suggest there may be some artefact to these measurements, possibly a UV illumination induced change. A necessary control for these experiments both to calibrate results to known published responses and to assess the possibility that changes observed are already baked into unstimulated cells is to perform measurements of the initial calcium signals induced in real time by TCR and CD3/CD28 in unstimulated freshly isolated cells over 15-30 minutes. The authors should then analyze differences or similarities. This would also speak to any possible pre-existing role for KCa channels in resting cells. Also, if EGR4 normally suppresses KCa expression why do WT cells exhibit a progressive increase in "steady state" levels and EGR4 -/- exhibit a transient increase and then decrease at 20 hours, while at 20 hours the levels of nuclear NFAT in WT and DKO are the same? It seems as if the interpretations here reflect expectations rather than a purely objective read of the data.
As stated above, we have examined TCR-induced Ca^{2+} signals in resting cells, and found no difference, countering the view that changes observed are already “baked into” unstimulated cells. We don’t fully understand the reviewer’s comment about a potential artefact in EGR1-/- measurements due to “UV illumination induced change”**.** Of note, WT, EGR1-/- and EGR4-/- cells were all treated the same way, and the experiment was performed several times with similar results. Hence, the differences in Ca^{2+} signals reflect genuine differences in the genotypes. Regarding the reviewer’s comment that our interpretations reflect our expectations, we observed increased potassium channel expression, Ca^{2+} channel activity, NFAT nuclear localization and IFNγ production in EGR4-/- cells, and established that pharmacological blockade of either of the first 2 steps blocks IFNγ production in EGR4-/- cells. Our interpretations are fully consistent with these data rather than with our expectations. The reviewer’s comment may reflect poor explanation on our part, and we have worked to improve our writing in the revised manuscript.

9) I am also confused by the EGR1 -/- results and wonder what the basis is for muted signals (2 and 20 hours). Would a further analysis of this inform a general mechanism of control over calcium by the EGRs? The authors never discuss the implications of this apparent inhibition of calcium signaling in EGR1 -/- cells. The authors should also confirm that BPT2 blocks NFAT localization in both WT and EGR4-/- cells to close the circle on this mechanism. Furthermore, although calcium levels are higher in EGR4 -/- cells, the delayed effect of BPT2 inhibition is perplexing and suggest that something else is at play here. Why does BPT2 not equally effect IL-2 production, which is a classic cytokine whose expression is exquisitely sensitive to calcium/NFAT. Why doesn't BPT2 fully suppress IL-2, not just the "hyperinduction". Why is a significant portion of IL-2 production in EGR4-/- cells calcium insensitive? Finally, as the goal of this experiment was to demonstrate cause and effect between EGR4 expression, calcium levels, and NFAT and differences are attributed to K channels, the authors should evaluate the impact of this blocker and the K channel inhibitors on NFAT localization.

We agree with the reviewer that we did not discuss our results in EGR1-/- cells in great detail and have added this discussion to our revised manuscript. In brief, we have previously shown that EGR1 drives STIM1 expression, which could definitely contribute to the muted response that the reviewer refers to. Regarding the effect of BTP2 on NFAT nuclear localization and IL2 production, it is important to recognize that this point is well established in the literature. Within the manuscript, we used low doses of BTP2 with the goal of assessing the possibility that we could attenuate excessive Ca^{2+} responses observed in EGR4-/- T cells without completely blocking T cell activation. These points have been clarified in the revised manuscript.

10) Given the established role for Kv1.3 in IL-2 production by and proliferation of T cells, why did the authors NOT test the effect Shk-Dap22 on calcium or proliferation to validate these mechanisms in WT cells and establish a basis for comparing a role in EGR4 -/- cells. At the very least these data would be an excellent positive control and Shk-Dap22's impact on WT and KO cells would more completely inform the mechanism of calcium regulation and its consequences. Indeed, the authors do NOT establish that elevated Kv1.3 is NOT involved in the increased proliferation of EGR4-/- cells.

Although certainly interesting, the proposed studies speak to mechanism, and as such are beyond the scope of the current study.

11) In general, the differences in tumor growth and metastasis in WT and EGR4-/- mice are intriguing and significant. The phenotype is convincing and well documented. However, the mechanistic analysis is less compelling. This reflects a general concern with this manuscript, that almost none of the FACS analysis in these studies is properly controlled and the gating strategies, and in some cases the gating is not validated or properly performed. This applies to all analysis of Tregs and FACS plots from which summarized analyses are shown in figures. For each subset, examples of primary FACS plots including the subset gating and controls used to establishing gates/regions should be included in the supplement. This is especially a concern for data in Figures 9G, H, I, and J (and this raises questions about the analysis in 9B) and in Figures 10 D-K, which also has an additional problem in that the gates are not identical for different genotypes. Additional concerns revolve around the analyses of FR4 and any conclusions drawn from these results. Without proper controls and explanation of a gating strategy, the FR4 analysis is incomprehensible. Consequently, conclusions about anergy based upon this analysis are weak. Furthemore, if anergy is really a factor, this should be evaluated in functional assays. Another conclusion that EGR4-/- cells have increased killing capacity could be tested directly and would strengthen this mechanism.

We apologize for deficiencies in how our gates were drawn in the first submission of our manuscript. Gates have been redrawn to ensure that they match precisely.

Referee #2:

Major points

1. Explanation of results obtained a few days after stimulation, despite Egr4 expression being both very transient, and at a very low level. It is consistent with previous findings; the author

has demonstrated a very transient and low level expression at mRNA levels in T cells following anti-CD3 stimulation in vitro. However, the majority of results obtained in vitro represent only a few days post-stimulation. If there is indeed the indirect effect of Egr4, authors must demonstrate the rescue effect of Egr4 by overexpression Egr4 into wild type or Egr4 KO T cells.
The reviewer appears to question whether T cell defects in EGR4ko mice are attributable to the EGR4 mutation itself, or due to a mutation at some unknown locus. This is highly unlikely, given that Egr4ko mice have been outcrossed to C57BL/6 mice for >10 generations, with mice at each generation selected for presence of the Egr4ko allele. Therefore, any mutation at an unknown locus would have segregated away from the Egr4 knockout locus in the meantime with very high probability. Furthermore, ectopic overexpression of Egr4 in T cells may dominantly affect T cell function, and may therefore not lead to proposed "rescue" of Egr4ko phenotype. Therefore, we believe such studies are beyond the scope of the present manuscript.

2. Lack of support for the Hyper-proliferation conclusion. Aside from TCRb stimulation, the proliferation results are less convincing. This may be due to the high doses of anti-CD3. A dose-dependent result and cell cycle analysis will give better results if Egr4-/- T cells are hyper-proliferative in response to TCR and costimulatory stimulation.
We completely agree with the reviewer and have provided a complete timecourse in revised figure 2, showing that EGR4-/- T cells are hyperproliferative in response to both weak and strong TCR signals.

3. Lack of mechanistic explanation for the over production of IFNg by CD4, and the increased effector function of CD8. Increased IFNg production and CD8 effector function have been reported from Egr2/3-/- T cells and these have resulted from the defect expression of repressors, uncontrolled T-bet activity and/or Lag3 function. Authors must assess the TFs defining Th1 and CD8 functions, as per stat5 and T-bet.
We are aware of the role of T-bet and Lag3 that has been described in EGR2/3-/- cells. In the current study, we establish that Ca^{2+} signals are required for Th1 bias in EGR4-/- T cells using both CRAC channel and K channel inhibitors. In addition, although we did not discuss it in the previous submission, no difference in T-bet expression is observed in EGR4-/- T cells. Based on editor's advice that further mechanistic work is not required and given that the manuscript is already "data-heavy", we do not include the result here.

4. RNAseq results are missing. TF function is translated by target genes, or genes that have been regulated indirectly.
We recognize that RNAseq could provide a great deal of information. However, our study includes 18 full length figures that establish a conceptual link between EGR4, potassium channel expression, Ca^{2+} signal generation and IFNγ production. As such, the addition of RNAseq data is beyond the scope of this study, according to the the Editor's advice.

5. Explanation of the cause of TCR signalling assays. Authors showed that Egr4 mRNA are not expressed until after anti-CD3 stimulation for hour or two, then rapidly retracted to background levels. However, TCR signalling is much more rapid than the induction of Egr4. How can we explain this?
We don't understand the basis for this question. The lag of 1 to 2 hrs between TCR engagement and peak EGR4 mRNA levels is consistent with all our functional readouts. Also, it's possible that absence of Egr4 at a prior stage, e.g. during development, may result in permanent changes in gene expression that affect even the earliest events in TCR signaling response.

6. Lack of mechanisms for supporting Treg. Although Foxp3+CD4 T cells are reduced, the development of Treg and their function in suppressing effector T cells are not examined.
In our opinion, such studies would be beyond the scope of the current study, and according to Editor's advice further mechanistic work is not required.

7. B16 is a melanoma model. We do not understand why B16 is used to investigate tumour metastasis. TIL are highly inflammatory, but the number of cases does not increase. Authors must examine proliferation markers, as terminally differentiated T cells are highly apoptotic.
We apologize for any confusion that we may have caused in the design of our experiment. We did not use B16 cells to examine tumor metastasis, but rather to induce an immune response *in vivo*.

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This is the most widely used model in the field, also used for immune therapy research (Cell, 2017, 170: 1120-1133). As outlined in our manuscript, significant differences in T cell responses to B16N cells were observed *in vivo*, consistent with our predictions.

8. Excessive production of IFNg in tumour microenvironments is associated with increased metastasis of the tumour. Authors should examine the tumour specificity of TIL by in vitro stimulation with tumour cells, or DCs loaded with tumour lysates. The hyper-inflammatory results from both TIL and T cells in spleens may indicate a response that is innate.
We agree with the reviewer that clear evidence that T cells are responsible for anti-tumor immunity was needed. These experiments have been repeated in Rag-/- mice after adoptive transfer of WT vs. EGR4-/- T cells (Fig EV5), firmly establishing that this is a T cell intrinsic phenotype.

9. The gating between the control and KO is not consistent in Fig. 9G, H, I, J and Fig. 10. J, K.
As stated in comments made in response to reviewer 1, we have adjusted the gating in these figures to match control and KO.

Referee #3:

In that sense, are the EGR4 effects on calcium alone responsible for the increased in proliferation and cytokine expression? If that is the case why is there only minimal effects on IL-2 expression compared to other cytokines (Fig. A-F) when IL-2 transcription is clearly dependent on calcium signaling? Are the frequency and amplitude of the modulation of calcium currents preferentially induce specific cytokines? If so how? Does EGR4 directly or indirectly repress the transcription of any of those genes?
We agree with the reviewer that IL2 expression is dependent on Ca^{2+} signals. It is important to recognize that the experiments performed in figure used very low doses of BTP2, with the goal of blocking Ca^{2+}-dependent responses in EGR4-/- cells, but not blocking T cell activation in general. As discussed in response to reviewer 1, the ability of BTP2 to block IL2 production is well established in the literature, as is the fact that BTP2 is only partially effective at the doses used. This is discussed further in the revised manuscript. Finally, as stated above, due to lack of an effective anti-Egr4 antibody, we cannot currently carry out ChIP-seq to identify direct targets of Egr4.

The increase in calcium entry is proposed to be likely mediated by the increased expression of two potassium channels. The experiments performed to prove this point are carried out using channel inhibitors. One would expect that no matter if the expression of those channels is elevated or not in Egr4-/- cells, blocking their function would have a inhibitory effect on calcium currents in any T cell. It would have been a much better approach, first to confirm that K currents are affected and then to use siRNA to silence the expression of those channels to levels as similar as possible to the ones found in wt cells.
We agree with the reviewer that a partial rather than complete blockade of K channel function is critical to establishing the link between K channels, Ca^{2+} responses and IFNγ production in EGR4-/- cells. However, in our opinion, the pharmacological strategy we have used is better suited than an siRNA-based strategy for establishing this point, given that siRNA-mediated knockdown of K^+ channels is not conducive to partial inhibition and may profoundly change the physiology of WT cells.

Reduced Treg differentiation is attributed to either an effect of the loss of EGR4 on Helios expression or to the consequences of increased expression of TNF in Egr4-/- T cells. However, none of these hypotheses are tested. Does blocking TNF restore Treg differentiation? Is Helios expression dependent on Egr4 (ChIP, reporter experiments...) or is it a secondary effect? The characterization of Th differentiation in Egr4-/- cells shows increased IFNg expression under all conditions w/o major differences in the production of other lineage defining cytokines. However, this data is presented only as secretion measured by ELISA, which prevents determining whether a pool of cells remains undifferentiated and expressing high levels of IFNg or if the absence of Egr4 leads to the generation of abnormally differentiated cells that express both IFNg and other cytokines. Intracellular staining for those cytokines would be necessary to answer this question.
In agreement with the reviewer, we have now performed intracellular staining for IFNg to better define the nature of the differentiative defect observed in EGR4-/- cells. Our data show that the

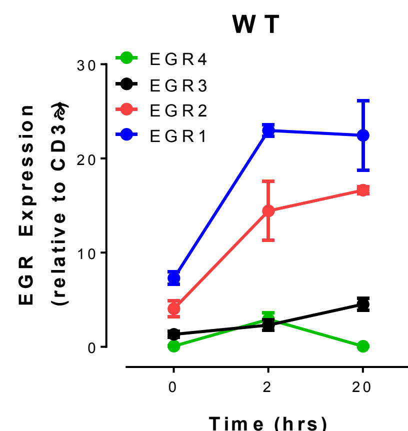
former hypothesis is correct; there are pools of undifferentiated and high IFNg-expressing cells (see figure 3 of the revised manuscript). As stated above, due to lack of Ab we cannot perform an EGR4-ChiP experiment.

The data from the tumor model is compelling but the model used is a full body KO and cannot conclusively prove that the better control of metastases seen in Egr4-/- mice responds to the development of increased Th1 like responses and/or to decreased Treg differentiation. In order to do so, in the absence of a floxed mouse, more specific approaches should be followed, such as BM chimeras spiking TCR-/- BM with EGR4-/- BM, or at least adoptive transfers of tumor antigen specific Egr4-/- T cells (e.g. TRP-1 system for B16) or even using surrogate antigens. It is also not clear why the i.v. injection model was used, which delivers a very high load of cells with metastatic potential, instead of a more physiologic model (e.g. B16F10) that would also allow evaluation of primary tumor control. There are also some concerns with the different results obtained with the IVIS system and the actual count of metastases. Are those discrepancies due to increased number but reduced size of lung metastases in the Egr4-/- mouse or are they just due to some technical limitation of the IVIS system? The authors keep referring to a role of Egr4 on thresholds of activation, and based those observations in the comparison of anti-CD3 or with anti-CD3 and anti-CD28 antibodies. Strictly speaking, these conditions do not represent different strengths of activation but rather the presence or absence of costimulation, and may support that effects seen may or may not be costimulation dependent. Analyses using different strengths of TCR stimuli would be needed to determine if Egr4 may control thresholds for activation.
We agree with the reviewer that clear evidence that T cells are responsible for anti-tumor immunity was needed. These experiments have been repeated in Rag-/- mice after adoptive transfer of WT vs. EGR4-/- T cells (Fig EV5), firmly establishing that this is a T cell intrinsic phenotype.

Minor points:

FACS gating is not offered in some analysis (e.g. Fig. 1). To better evaluate the characteristics of all cell populations analyzed, sample flow graphs with gating strategy should be provided at least as Suppl. Data.
Agreed and corrected.

Although it might be due to the conversion to pdf, the quality of the IF in Fig4 is quite low. In any case the differences in NFAT nuclear translocation, though statistically significant, are low in magnitude. Are they biological relevant? Can they explain the marked changes in cytokine expression and activation-induced proliferation seen in Egr4-/- T cells? Modulation of EGR1-3 expression in WT cells as shown in Fig. 1 is obscured by the huge compensatory increases that occur in EGR deficient mice. As one of the important points made by the authors to explain non-redundant functions of EGR4 and other EGR protein, is that their kinetics of expression are very different (biphasic vs sustained), it would make it easier for the reader to appreciate those differences if graphs showing only the regulation of Egr gene expression in wt cells was also offered.
As requested by the reviewer, we have provided data showing EGR expression in WT cells. However, given it's length, we do not think that it would be appropriate to duplicate data in the actual manuscript.



The authors claim that "However, there was a slight but statistically significant increase in naïve CD4+ and CD8+ T subsets in EGR4-/- mice (Fig.1E)" However, there are no such stats shown for naive CD8+ T cells. Furthermore, the reference should be to Fig 1F-G.
Apologies for our error; there is no difference in CD4 and CD8 subset numbers. The manuscript has been revised to reflect this and figure reference numbers have been corrected.

Should Fig 1K be a dot plot?

No, however, we have corrected our error in the labelling of the Y-axis to indicate cell number.

2nd Editorial Decision 21 February 2020

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Nevertheless, referees #1 (referee #3 from the submission to TEJ) and referee #2 (referee #1 from the TEJ submission) have several remaining concerns and suggestions to improve the manuscript, I ask you to address in a final revised version of the manuscript. Please also provide a detailed point-by-point response that addresses the remaining points of the referees.

Further, I have these editorial requests I ask you to address:

- Please add up to 5 key words to the title page of the manuscript.

- We will publish the manuscript in the article format. This requires that results and discussion sections are separated. Please do that for your manuscript. See also: https://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- Please remove the list of abbreviations. Please define any abbreviation upon first mention in the manuscript text.

- Please add a formal "Data Availability section" (placed after Materials & Methods) to the manuscript. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

- Please significantly reduce the number of panels in the figures. I think several panels need to be combined (e.g. in Fig. 8 with panels A-Y!). Please indicate differences by respective labelling in the figure or define these in the legends. It also seems that some of these panels are presently not separately called out (e.g. Fig. 8 panels I-L).

- There seem also to be no call-outs for Figs. 2I, 3D, 6E and the Appendix Figs. S7 and S8. However, there is a call-out for a Fig. 10R-X (please fix). Please check that all panels are called out, and are correctly called out.

- Please add page numbers and a table of contents (TOC) with page numbers to the Appendix. The Appendix figures need to be named "Appendix Figure SX". Please do that and the change the call out for these in the manuscript text. Please make sure that all the Appendix figures are called out.

- Please make sure that regarding data quantification and statistics, the number "n" for how many independent experiments (biological vs technical replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is specified in the respective figure legends (ALSO in the Appendix). Please provide statistical testing where applicable, AND also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

- Please change the sentence "... with significant differences marked by *" to "... with significant differences marked by asterisks". I think that looks less confusion.

- It seems the contributions of the authors Emmanuelle Nicolas and M Raza Zaida are missing from the author contributions. Please add these.

- Figure 1J shows up again in Appendix Fig. S1B (middle left). Please explain, and if this is intentional (which I assume) please mention this in the respective figure legends.

- We require that all corresponding authors supply an ORCID ID for their name. This is still missing

for Dietmar Kappes. We will not proceed before this is done. Please find instructions on how to link his ORCID ID his account in our manuscript tracking system in our Author guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

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

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

REFEREE REPORTS

Referee #1:

In this revised version of the manuscript by Mookerjee-Basu et al., the authors have addressed most of the concerns raised in the previous round on review and present now a convincing story that identifies a new role for EGR4 in the regulation of T cell differentiation, likely through the modulation of the amplitudes and characteristic of the K/Ca currents induced in response to TCR engagement. New analyses have been performed to more conclusively prove how EGR4 regulates cytokine secretion and T cell differentiation, justifications have been provided for some of the experimental approached used and, importantly, BM chimeras and adoptive T cell transfer have been used to show a T cell intrinsic role of EGR4 in the observed dramatic differences in the response to B16 in mice lacking this TF. Some of the proposed mechanisms that explain how EGR4 may regulate T cell activation are still not fully explained. For instance, the role of K channels while supported by the inhibitor experiments would need to be proven more conclusively: i.e. does the increased expression of those channels lead to changes in K currents or intracellular levels of potassium? Can the effects seen observed in EGR4-/- T cells be phenocopied at any level by overexpression of those channels? Also, the issue of the selective effect of those changes on Th1 differentiation and IFNg expression is left without being addressed. However, I think that the novelty of this research and its potential significance, as well and the compelling data that supports that EGR4 regulates T cell function and promotes Th1 responses justifies its publication.

There are, however, still a few minor points that I think the authors may want to address.

The authors claim that they "observed strong but transient induction of EGR4 beginning at 2 hrs of activation in WT CD4+ T cells (Fig 1D)." and based on that observation proposed that this induction of EGR4 may be involved in the regulation of T cell responses. This increased is however quite small (2-3-fold at the mRNA levels without currently the ability to see if this translates into changes in protein levels) and according to Fig 1D not statistically significant. There are however compelling data in the paper that, as pointed in previous comments, the lack of EGR4 may change the steady state of CD4+ T cells and poise them to respond more strongly and with a Th1 bias. For instance, in the absence of any stimulation cells lacking EGR4 already are able to produce significant amounts of several cytokines, and the increase in the expression of genes coding for the K channel is already upregulated in resting cells. All these data give a strong support to this alternative explanation and it may be worth to include it as a possibility in the manuscript.

In the conclusions the authors state that: "Notably, the use of either potassium channel or SOCE inhibitors blocks both EGR4-dependent increases in Ca2+ signals and increased IFNγ production in EGR4-/-, but not in WT T cells" While this is true for SOCE inhibitors, the data shown in Fig. 6E-F clearly shows that K channel inhibitors were also able to reduce IFNg expression in WT cells and in the case of Senicapoc as effectively or even more than in EGR4-/- cells. This statement should be corrected.

Figs 3 G and H offer only representative FACS plots, but no quantification of multiple experiments is provided.

The authors state that "Both EGR4- and EGR1-deficient CD4+ T cells initially showed increased proportions of IFNg-producing cells under Th1 polarizing conditions compared to WT cells (Fig 3H) after 3 days, but equivalent levels to WT cells by 5 days post-stimulation (Fig 3I)" However while Fig. 3H is shows data on the number of cells that can produce IFNg, Fig. 3I does not. The data in Fig. 3I show ELISA data and it is difficult to compare to the data presented in 3I. This statement could be modified to indicate the difference in the nature of data in each figure.

Referee #2:

General Comments:

This revised manuscript by Mookerjee-Basu addresses most of the issues raised previously by this reviewer. I am still confused by the argument that calcium levels remain elevated in EFR4-/- cells at later timepoints simply due to changes in K channel expression. This does not make sense physiologically to me. The results imply something more is going on. While membrane potential could surely play an important role in sustaining calcium entry, an underlying necessity is that Orai channels are also hyperactivated. Further, the timing is apparently different for EGR4-/- cells (max at 2 hours) versus WT cells (20 hours). The mechanistic basis for these differences are not adequately addressed experimentally or discussed. It would be helpful to provide some explanation for what seems apparent, that CRAC channels remain activated for long time periods. Is there a plausible explanation for this based upon what is known or can the authors speculate?

Minor Comments:

1. Description of Fig 1A to 1D needs to be tightened. EGR expression was not increased "beginning" at 2 hours, that's the first time point observed. Also, the following statement is not an accurate characterization of the results shown, "Thus, EGR1, EGR2 and EGR3 expression persisted and/or increased over time up to 20 hrs" and statistics provided.

2. The authors state that EGR4 expression, unlike EGR1-3, is transient but fail to show statistics to demonstrate a significant increase in EGR1-3 at 2 and 20 hours. Given the apparently small change, these statistics are needed.

3. The authors report no significant decrease in CD8+ CM T cells relative to WT cells. Why is this not a concern? There is a significant increase in CD4+ CM cells. Further, the manner of labeling significance (a, b, c) is unclear and confusing and the criteria/cutoff for significance is not provided.

4. Figure 2. Neither the methods, results, nor legends provide a clear picture of how this analysis was performed, so this needs clarification. I suspect more is going on here than revealed by the authors presentation as they do not perform absolute cell counts or assess apoptosis. The data presented could reflect the fact that EGF4-/- cells are undergoing apoptosis at a greater rate than WT cells. This is another reason to show CFSE vs Live/dead profiles for each condition.

5. On p.11 (and elsewhere) the authors mistakenly refer to an increase in calcium OSCILLATIONS in EGR4-/- cells ("Given our prior findings that EGR1 and EGR4 regulate STIM1 [26, 55] and PMCA4 [26] expression, our initial hypothesis for why EGR4-/- T cells exhibit increased Ca2+ oscillations was that EGR4 deletion led to dysregulation of STIM and/or PMCA expression". None of the calcium signals can be characterized as OSCILLATION. Furthermore, the authors do not demonstrate or quantify the signals in terms of periodicity and they do not exhibit the characteristics of "oscillations". The language should be modified throughout the manuscript to modified accordingly.

6. On page 12 the authors state, "As depicted in figure 6, blocking Ca2+ entry with BTP2 blocked the increased IFNγ production characteristic of EGR4-/- T cells. Since KCa3.1 activity is required for IFNγ production, we would expect that blocking KCa3.1 would lead to the same result. As depicted in figure 6F and 6G, that is exactly the case. We then tested cells with the Kv1.3 inhibitor Shk-Dap22, observing a similar potent inhibition of IFNγ production (Fig 6G), suggesting redundancy between these 2 channels." The references to panels E, F, and G seem to be incorrect

and furthermore no experiments with BTP2 are shown in Figure 6.

7. No stats for figure 7

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Referee #3:

The manuscript has been improved with additional data. Although the mechanisms of Egr4 mediated function are still not clear, the Egr4 function in T cells is novel.

2nd Revision - authors' response 24 February 2020

Referee #1:

The authors claim that they "observed strong but transient induction of EGR4 beginning at 2 hrs of activation in WT CD4+ T cells (Fig 1D)." and based on that observation proposed that this induction of EGR4 may be involved in the regulation of T cell responses. This increase is however quite small (2-3-fold at the mRNA levels without currently the ability to see if this translates into changes in protein levels) and according to Fig 1D not statistically significant.
We apologize for the complexity of this figure and associated statistics. The statement that the increases in EGR4 expression was only 2-3 fold is not quite accurate; since EGR4 is virtually not expressed in WT T cells, the increase in EGR4 expression was ~30-fold. Further, 2-way ANOVA reveals significantly different EGR4 expression over time.

There are however compelling data in the paper that, as pointed in previous comments, the lack of EGR4 may change the steady state of CD4+ T cells and poise them to respond more strongly and with a Th1 bias. For instance, in the absence of any stimulation cells lacking EGR4 already are able to produce significant amounts of several cytokines, and the increase in the expression of genes coding for the K channel is already upregulated in resting cells. All these data give a strong support to this alternative explanation and it may be worth to include it as a possibility in the manuscript.
Within the current version of the manuscript, we discuss the potential role of EGR4 in making T cells poised.

In the conclusions the authors state that: "Notably, the use of either potassium channel or SOCE inhibitors blocks both EGR4-dependent increases in Ca2+ signals and increased IFNγ production in EGR4-/-, but not in WT T cells" While this is true for SOCE inhibitors, the data shown in Fig. 6E-F clearly shows that K channel inhibitors were also able to reduce IFNg expression in WT cells and in the case of Senicapoc as effectively or even more than in EGR4-/- cells. This statement should be corrected.
Done.

Figs 3 G and H offer only representative FACS plots, but no quantification of multiple experiments is provided.
Quantitation has now been added to the figure, showing statistically significant differences.

The authors state that "Both EGR4- and EGR1-deficient CD4+ T cells initially showed increased proportions of IFNg-producing cells under Th1 polarizing conditions compared to WT cells (Fig 3H) after 3 days, but equivalent levels to WT cells by 5 days post-stimulation (Fig 3I)" However while Fig. 3H is shows data on the number of cells that can produce IFNg, Fig. 3I does not. The data in Fig. 3I show ELISA data and it is difficult to compare to the data presented in 3I. This statement could be modified to indicate the difference in the nature of data in each figure.

Apologies for the confusion; the panels were incorrectly references as 3H and 3I instead of 3G and 3H. Corrected.

Referee #2:

General Comments:

This revised manuscript by Mookerjee-Basu addresses most of the issues raised previously by this reviewer. I am still confused by the argument that calcium levels remain elevated in EGR4-/- cells at later timepoints simply due to changes in K channel expression. This does not make sense physiologically to me. The results imply something more is going on. While membrane potential could surely play an important role in sustaining calcium entry, an underlying necessity is that Orai channels are also hyperactivated. Further, the timing is apparently different for EGR4-/- cells (max at 2 hours) versus WT cells (20 hours). The mechanistic basis for these differences are not adequately addressed experimentally or discussed. It would be helpful to provide some explanation for what seems apparent, that CRAC channels remain activated for long time periods. Is there a plausible explanation for this based upon what is known or can the authors speculate?
We agree with the reviewer that K channel activity alone cannot account for this phenotype. By necessity, TCR-mediated PLC activity and subsequent Orai1 activation are necessary for this response to occur. Our theory is that increased K channel activity changes the scale of the ensuing Ca^{2+} response. Regarding the change in time course, we would speculate that the relatively fast termination of the Ca^{2+} response in EGR4-/- cells may reflect the early onset of the Ca^{2+} response ie the duration of the Ca^{2+} response may be self-limiting. Speculations to this effect have been added to the discussion.

Minor Comments:
1. Description of Fig 1A to 1D needs to be tightened. EGR expression was not increased "beginning" at 2 hours, that's the first time point observed. Also, the following statement is not an accurate characterization of the results shown, "Thus, EGR1, EGR2 and EGR3 expression persisted and/or increased over time up to 20 hrs" and statistics provided.
Corrected.

2. The authors state that EGR4 expression, unlike EGR1-3, is transient but fail to show statistics to demonstrate a significant increase in EGR1-3 at 2 and 20 hours. Given the apparently small change, these statistics are needed.
Please note that only post-hoc differences are marked on the graph. The results of our two way ANOVA are reported within the figure legend, revealing significant changes in EGR expression over time.

3. The authors report no significant decrease in CD8+ CM T cells relative to WT cells. Why is this not a concern? There is a significant increase in CD4+ CM cells. Further, the manner of labeling significance (a, b, c) is unclear and confusing and the criteria/cutoff for significance is not provided.
The system for labelling significance is now defined in the methods section; $p < 0.05$ is the cutoff for significance between groups. We are not clear what the concern of the reviewer is regarding whether or not there are significant changes in CM T cells.

4. Figure 2. Neither the methods, results, nor legends provide a clear picture of how this analysis was performed, so this needs clarification. I suspect more is going on here than revealed by the authors presentation as they do not perform absolute cell counts or assess apoptosis. The data presented could reflect the fact that EGR4-/- cells are undergoing apoptosis at a greater rate than WT cells. This is another reason to show CFSE vs Live/dead profiles for each condition.
For CFSE dilution studies, dead cells were stained with Propidium Iodide and gated out prior to calculating the Proliferation Index. As such, all data reflects live cells only. The methods section has been modified to reflect this new information.

5. On p.11 (and elsewhere) the authors mistakenly refer to an increase in calcium OSCILLATIONS in EGR4-/- cells ("Given our prior findings that EGR1 and EGR4 regulate STIM1 [26, 55] and PMCA4 [26] expression, our initial hypothesis for why EGR4-/- T cells exhibit increased Ca2+ oscillations was that EGR4 deletion led to dysregulation of STIM and/or PMCA expression". None of the calcium signals can be characterized as OSCILLATION. Furthermore, the authors do not demonstrate or quantify the signals in terms of periodicity and they do not exhibit the characteristics of "oscillations". The language should be modified throughout the manuscript to modified accordingly.
Corrected as requested.

6. On page 12 the authors state, "As depicted in figure 6, blocking Ca2+ entry with BTP2 blocked the increased IFNγ production characteristic of EGR4-/- T cells. Since KCa3.1 activity is required for IFNγ production, we would expect that blocking KCa3.1 would lead to the same result. As depicted in figure 6F and 6G, that is exactly the case. We then tested cells with the Kv1.3 inhibitor Shk-Dap22, observing a similar potent inhibition of IFNγ production (Fig 6G), suggesting redundancy between these 2 channels." The references to panels E, F, and G seem to be incorrect and furthermore no experiments with BTP2 are shown in Figure 6.
Inaccurate figure references have been corrected.

7. No stats for figure 7
There are in fact statistics in figure 7.

8. There seems to be a problem with figures 8A-D, "In contrast, little or no differences in IL-9 or IL-17 producing cells were observed in lymphocytes collected from either tumors or spleens" This is not evident. Also, figure 7I-L legends are missing.
The statement and legend have been corrected.

Referee #3:

The manuscript has been improved with additional data. Although the mechanisms of Egr4 mediated function are still not clear, the Egr4 function in T cells is novel.
Thank you for your comments.

Accepted 27 February 2020

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Experiments were performed a minimum of 3 times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Experiments were performed a minimum of 3 times.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Data was not excluded from analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No formal steps to randomize were taken.
For animal studies, include a statement about randomization even if no randomization was used.	No formal steps to randomize were taken.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Experiments were not blinded.
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5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Statistics were performed and validated in Graph Pad Prism

Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All experiments included internal controls to validate antibodies
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We did not use cell lines.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Experiments were performed on C57 Bl/6 mice (2 to 6 months). Both male and female mice were used. We compared WT, EGR1-/- and EGR4-/- mice. Animals were derived from crosses of heterozygous parents and housed with 1 to 4 same sex, genotype matched animals.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Our ULAR facility is AALAC acredited.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Our ULAR facility is AALAC acredited.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No human subjects
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	No human subjects
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14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No human subjects
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No sequencing or structural data.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Understood.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Understood.
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G- Dual use research of concern

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