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Regulatory T cells suppress CD4+ T cells through NFAT-dependent transcriptional mechanisms

Daniel Shin, Ayana Jordan, Samik Basu, Rajan M Thomas, Sanmay Bandyopadhyay, Edwin F de Zoeten, Andrew D Wells and Fernando Macian

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

Editor: Nonia Pariente

1st Editorial Decision 10 December 2013

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest, they all raise serious concerns regarding the conclusiveness of the data and point out several technical issues that prevent a solid interpretation of the evidence provided. All of the reviewers call for a considerable amount of additional experimentation to resolve these issues.

From the analysis of these comments it is clear that we cannot consider the publication of your manuscript at this stage, and that the work needed goes beyond what would normally be considered for a revision. However, given the potential interest of the findings, we would like to give you the opportunity to revise your manuscript.

After going through the reports in detail and further discussions with the referees, it is clear that several issues must be addressed for the study to become suitable for publication in EMBO reports. Given the emphasis of EMBO reports in functional and physiological relevance over detailed mechanism, the physiological relevance of the findings needs to be evaluated, by using for example a colitis model. Importantly, all referees have problems with the data shown in figures 1 and 2, and these would need strengthening by using a conventional suppression assay, CSFE labeling and ideally a readout other than IL-2. The experiment in figure 1 would need to be performed in

congenic mice and measuring absolute cell numbers, and in figure 2 the use of BrdU was considered problematic. The epigenetic regulation of IL-2 is insufficiently shown, and needs to either be strengthened or perhaps excluded from the study.

There are additional minor issues which would be good to address. However, two specific issues would be out of the scope of the present study and do not need to be pursued: the regulation of iTregs and the chimeric experiments suggested by referee 2.

If the referee concerns can be adequately addressed, we would be happy to consider your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review. I realize that fully addressing the referee concerns along the lines indicated above would involve a lot of additional experimental work and I am unsure if you will be able to return a revised manuscript within our 3 month deadline. I could potentially reasonably extend this period (should you feel time would be the only limitation to a successful revision of the paper), but would also understand if you rather chose to seek rapid publication elsewhere at this stage.

Should you decide to embark on such a revision, revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. Revised manuscript length must be a maximum of 28,500 characters (including spaces). When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees.

I look forward to seeing a revised form of your manuscript when it is ready. If you decide to submit the study elsewhere, please let us know. Do not hesitate to get in touch with me if I can be of any help.

REFEREE REPORTS:

Referee #1:

Much effort has been devoted to delineate the inhibitory mechanisms through which Treg cells enforce immune tolerance by suppressing conventional T cells and other immune cells. Most of the studies have focused on the Treg, the suppressor, side. The knowledge on the suppressed side, especially the responding conventional T cells, is less well documented. Nonetheless, a number of recent studies have revealed some of mysteries in the suppressed T cells, such as enforced senescence (Ye J, et al, Blood 2012; 120: 2021), conversion to suppressor cells (Collison LW, et al, Nat Immunol 2010; 11: 1093) and further potentiation of Treg function (Delgoffe GM, et al, Nature 2013; 501: 252). The manuscript by Shin D et al. expands Treg's arsenal by proposing that Treg cells co-opt NFAT1-Ikaros pathway, an important anergy mechanism, to suppress T cell activation. NFAT1 is known to induce Foxp3 expression and interact with Foxp3 to promote Treg function. Therefore, this study identifies NFAT1 as an overarching factor that maintains immune tolerance, both in Treg cells and in conventional T cells. How Treg activates NFAT1-Ikaros pathway, but not NFAT1-AP1 pathway, is an important question awaiting further investigation. Overall, the experiments are well designed and executed and the results are clear. I have the following comments.

1. Although the authors have provided nice mechanistic data, the physiological significance of their findings is less clear. The authors should strengthen this by including more in vivo studies, for example, by using the T cell transfer model of colitis that involves the co-transfers of naïve and Treg cells.
2. Figure 1F has technical issues that prevent a proper interpretation. First, separation of T cells from other cells is not optimal. Second, the gating of CD4+ cells is not sufficient to define donor T cells, as other cells in Rag KO mice could also express CD4; thus, anti-TCR or anti-CD3 antibody should be included to accurately define the donor cells. Third, and more importantly, the relative CD4+ percentage is not a good indication of T cell homeostatic expansion. Instead, the authors should present total CD4+ T cell number in the spleen. It will be even better if they can label donor naïve T cells with CFSE and present CFSE dilution in the presence or absence of co-transferred Treg cells.

3. The authors used differentiated Th1 cells to perform suppression assays. As NFAT1 deficiency perturbs Th1 vs Th2 differentiation, they should include suppression assays using WT and NFAT1-deficient naïve T cells.
4. As the authors showed in their previous study, NFAT1 mediates T cell anergy by affecting IL-2 locus epigenetic modification. They should examine whether Treg induce similar epigenetic changes in suppressed T cells.
5. It is interesting that iTreg-mediated suppression is independent of NFAT1 activity. The authors should test whether iTreg cells induce NFAT1 nuclear translocation in suppressed T cells.

Referee #2:

There are interesting observations in this manuscript, which claims that upregulation of Ikaros in CD4+ T cells-downstream of NFAT1 signaling-inhibits their activation in the presence of Treg cells. However, numerous issues prevent me from recommending this manuscript or publication in its current form.

The in vitro suppression assay used for most of the experiments in this manuscript is very particular, involving the culturing of Th1 clones with Treg cells that have been pre-activated with anti-CD3 and anti-CD28, and using diminished IL2 production as readout of suppression. The authors should consider exploring whether or not their observations hold true in the context of the conventional widely used in vitro suppression assay of proliferation of naïve CD4+ T cells cultured in the presence or absence of Treg cells.

Figure 1: is in vitro proliferation of naïve Nfat1-/- CD4+ T cells suppressed in the presence of Treg cells? For part F of Figure 1, can this data be shown in a clearer way? Does the CD4+ cell gate shown in the FACS plots include the transferred Treg cells? Are any of the transferred populations congenically marked? Absolute cell counts are needed to make sense out of these results.

Furthermore, can this in vivo experiment be done in a different way: it might be interesting to make a 1:1 Nfat1+/+ and Nfat1-/- bone marrow chimera and try to show that the CD4+ T cells arising from the Nfat1-/- compartment are hyperactivated compared to the Nfat+/+ CD4+ T cells.

In Figure 2G, the use of BrdU MFI as a readout of proliferation seems odd. The difference in MFI is used to calculate a percent inhibition. These experiments should be redone using [3H] thymidine incorporation or dilution of CTV or CFSE to make the result would be more convincing.

The 1998 Thornton and Shevach JEM paper describing the original in vitro suppression assay showed that anti-CTLA4 antibody had no effect on the ability of Treg cells to suppress. However, Figure 3E shows that CTLA4 inhibition restores full IL2 production by Th1 clones. Is there a disconnect between IL2 production and proliferation, or is this a difference between using Th1 clones and naïve CD4+ T cells in the suppression assay? Why are different results observed for iTreg cells?

In Figure 4B, the authors perform a conventional in vitro suppression assay. What happens in this assay to Nfat1-/- CD4+ T cells? It seems that previous publications have described abnormal CD4+ T cell development in in DN-Ikaros mice. To show that the effects seen in Figure 4 are not due to developmental alterations, can the authors retrovirally express DN-Ikaros in WT CD4+ T cells, similar to expression of Nfat1 in Nfat1-/- cells in Figure 1?

Also, the authors seem to be suggesting that Ikaros may mediate epigenetic changes at the IL2 locus. However, Figure 3 demonstrates that IL2 inhibition is not stable and that suppressed CD4+ T cells that have been rested for 4 hours reacquire the ability to make IL2.

Referee #3:

This report by Shin et al., links NFAT1 and IKAROS to the suppression of helper T cell IL-2 production by Treg cells. This ability if Tregs to use NFAT1 and IKAROS for mediating

suppression of IL-2 production was restricted the natural Treg cells; not induced Treg cells. The data to a certain extent supports the conclusions drawn however there are concerns with some of the experimental designs, presentation of data and more dogmatic conclusions.
1. Figure 1: it is not clear why the authors move between two different OVA-based readout systems, when the vast majority of the work is based in the DO11.10 system. This is confusing, particularly when different presentation styles are used for data analysis. For example in Figure 1A and B, both are supposed to be readouts for IL-2 levels using ELISA, yet where (A) gives no indication of ratios of Treg:Effectors and uses ng/ml concentrations for readouts (B) uses titrations and a relative IL-2 production levels. Consistency in readouts would enhance this figure.
Figure 1F. The figure legend does not make it clear how this assay was performed. Since both the effectors and Tregs transferred are CD4+ cells, how did the authors distinguish between the two. Potentially, transferred Treg cells could have lost Foxp3 expression and thus Tregs themselves could be incorporated in the readout. Similarly, not all Foxp3+ Treg cells are CD25+. This experiment needs performed using congenic mice and the absolute numbers of recovered effector CD4+ T cells need to be measured, not frequency.
Figure 1G, the rationale for this experiment- that NFAT1-deficiency may influence thymic development or differentiation of T helper cells- is confusing as the previous figures clearly show NFAT1-deficient T helper cells can produce IL-2 when appropriately stimulated. This experiment would be sufficiently pre-fixed by simply stating 'to confirm deficiency in NFAT1 contributes to inability of T helper cells to be suppressed by Tregs, we transduced....'.
Figure 2; again the use of two different OVA-based systems leads to confusion. In 2A, since GRAIL was not to be followed up, it may be best to leave that data out (not shown) and focus on IKAROS. Although it may seem a minor point, swapping Fig 2F with 2E would make the data flow better and emphasis an interesting finding that although IL-2 suppression still occurred with iTregs present, IKAROS upregulation was impaired.
Fig 2G was very confusing. Normally effector cells are labelled with CFSE, transferred into mice with Treg cells (of another congenic strain) and then CFSE-dilution is monitored only in effector cells. In this case it is very difficult to tell what cells the BrdU is being assessed in as the gating system in not transparent. Also there seems to be little evidence that the effector cells were suppressed to any extent based on the FACS plots shown.
Fig 3A and B, both figures were challenging to interpret due to their small size. Figures D-G add nothing to the manuscript as the data presented only reproduces well known aspects of potential Treg suppression mechanisms and since this manuscript provides no evidence that there is a direct link between NFAT1, IKAROS and loss of co-stimulation, then the authors should consider removing this data. This would free up space to expand the histological figures thereby enable better clarity for interpretation.
In this section, abstract and introduction, the authors go into detail that NFAT1 acts independent of AP-1 to control effector cells via Treg cell suppression. However, not one experiment is conducted that looks at AP-1. These comments therefore need removed.

Minor points:
There are a few typos throughout the manuscript.
Abstract, 4th line, the word 'by' is missing.
Introduction, page 3, 6 line down, this sentence would read better as derived Treg cells in the thymus (tTregs).
Results/discussion page 8, 8th line from bottom, suppressed should be suppress.
Page 9, first paragraph. Since the data is not being shown, the authors should state that here.
Page 10, Fig 4C is actually Fig 3C.
Page 13, this reviewer does not think the data presented warrants the rather strong statement of 'this detailed understanding'. The data is nice, but far from detailed. This comment needs toning down.

1st Revision - authors' response 30 April 2014

Point by point response to reviewers' comments

Referee #1:

We are glad that this this reviewer found that "…*this study identifies NFAT1 as an overarching factor that maintains immune tolerance, both in Treg cells and in conventional T cells. How Treg activates NFAT1-Ikaros pathway, but not NFAT1-AP1 pathway, is an important question awaiting further investigation. Overall, the experiments are well designed and executed and the results are clear."*

Specific comments have been addressed as follows:

1. Although the authors have provided nice mechanistic data, the physiological significance of their findings is less clear. The authors should strengthen this by including more in vivo studies, for example, by using the T cell transfer model of colitis that involves the co-transfers of naïve and Treg cells.

This is a very relevant observation. As we discussed in our original manuscript the model of Treg-regulation of colitis was not amenable to be used with NAFT1-deficidnt cells, as we could not induce colitis by transferring NAFT1-deficient T cells, likely due to the Th2 bias that is observed in those cells. This is why we decided to use the model a Treg-mediated inhibition of homeostatic proliferation with those cells. However, we were able to use the colitis method using T cells expressing a dominant negative form of Ikaros. Using these cells we now show that Tregs are much less efficient at inhibiting wasting induced by transferring naïve cells that express the dominant negative Ikaros into Rag1-/- hosts compared to their suppressive effect on wildtype cells. These new results are now shown in Fig. 4C.

2. Figure 1F has technical issues that prevent a proper interpretation. First, separation of T cells from other cells is not optimal. Second, the gating of CD4+ cells is not sufficient to define donor T cells, as other cells in Rag KO mice could also express CD4; thus, anti-TCR or anti-CD3 antibody should be included to accurately define the donor cells. Third, and more importantly, the relative CD4+ percentage is not a good indication of T cell homeostatic expansion. Instead, the authors should present total CD4+ T cell number in the spleen. It will be even better if they can label donor naïve T cells with CFSE and present CFSE dilution in the presence or absence of co-transferred Treg cells.

We have followed the reviewer's recommendations and have repeated those experiments transferring Th1.2 naïve T cells and Thy1.1 Treg cells into Thy1.2 Rag1-/- hosts. Transferred donor T cells are now identified as CD4+CD3+Thy1.1-. Absolute numbers of cells are also provided in the new Fig. 1G.

3. The authors used differentiated Th1 cells to perform suppression assays. As NFAT1 deficiency perturbs Th1 vs Th2 differentiation, they should include suppression assays using WT and NFAT1-deficient naïve T cells.

We have included analysis using naïve NFAT1-/- T cells in Fig. 1F, and show that they are also more resistant to Treg-mediated suppression than wildtype cells.

4. As the authors showed in their previous study, NFAT1 mediates T cell anergy by affecting IL-2 locus epigenetic modification. They should examine whether Treg induce similar epigenetic changes in suppressed T cells.

This was a very interesting idea as it could explain the differences we saw in the stability of the anergic phenotype compared with that of suppressed cells. Our new data (Fig. 2F) shows that Tregs fail to induce a significant reduction in histone acetylation at the IL2 promoter. This could account for the fact that the effect of Ikaros on suppressed cells is transient as it does not lead to stable epigenetic modifications. It is possible that other chromatin-remodeling proteins are also activated in anergic cells to cooperate with Ikaros in closing the IL2 locus and that these mechanisms are not fully activated in suppressed T cells. We are now continuing this line of research to understand better the exact epigenetic mechanisms that may explain the differences between the long-lasting inhibition of Il2 expression in anergic and the less stable inhibition in suppressed cells.

5. It is interesting that iTreg-mediated suppression is independent of NFAT1 activity. The authors should test whether iTreg cells induce NFAT1 nuclear translocation in suppressed T cells.

Our data suggest that iTreg-mediated suppression is NAFT-independent but other than that we still do not know much about how iTregs suppressed T helper cells. We decided, thus, to focus this report on describing the process that accounts for tTreg-mediated suppression of CD4+ T cells, although we currently actively pursuing the identification of the mechanisms that are activated in T cells when suppressed by iTregs.

Referee #2:

We are glad the reviewer found that *"There are interesting observations in this manuscript, which claims that upregulation of Ikaros in CD4+ T cells-downstream of NFAT1 signaling-inhibits their activation in the presence of Treg cells"*

The concerns raised have been specifically addressed as follows:

The in vitro suppression assay used for most of the experiments in this manuscript is very particular, involving the culturing of Th1 clones with Treg cells that have been pre-activated with anti-CD3 and anti-CD28, and using diminished IL2 production as readout of suppression. The authors should consider exploring whether or not their observations hold true in the context of the conventional widely used in vitro suppression assay of proliferation of naïve CD4+ T cells cultured in the presence or absence of Treg cells. Figure 1: is in vitro proliferation of naïve Nfat1-/- CD4+ T cells suppressed in the presence of Treg cells?
This very valid point that was also raised by referee #1. As we discussed above, we have included analysis using a CFSE dilution with naïve NFAT1-/- T cells (Fig. 1F) and show that they are also more resistant to Treg-mediated suppression than wildtype cells assay.

For part F of Figure 1, can this data be shown in a clearer way? Does the CD4+ cell gate shown in the FACS plots include the transferred Treg cells? Are any of the transferred populations congenically marked? Absolute cell counts are needed to make sense out of these results.

We have repeated those experiments using congenic markers to address this issue. Fig. 1G shows experiments carried out by transferring Th1.2 naïve T cells and Thy1.1 Treg cells into Thy1.2 Rag1-/- hosts. Transferred donor T cells are now identified as CD4+CD3+Thy1.1-. Absolute numbers of cells are now also provided

Furthermore, can this in vivo experiment be done in a different way: it might be interesting to make a 1:1 Nfat1+/+ and Nfat1-/- bone marrow chimera and try to show that the CD4+ T cells arising from the Nfat1-/- compartment are hyperactivated compared to the Nfat+/+ CD4+ T cells.

We also thought of this approach but given that NFAT1-/- T cells also deficient in the response to anergizing stimuli [1], it would be difficult to assign how much of the phenotype is due to reduced anergy and how much to reduced suppression, this is why we decided to use the adoptive transferred approach to better pinpoint differences in responses to Tregs.

In Figure 2G, the use of BrdU MFI as a readout of proliferation seems odd. The difference in MFI is used to calculate a percent inhibition. These experiments should be redone using [3H] thymidine incorporation or dilution of CTV or CFSE to make the result would be more convincing.

We agree with the reviewer that this might not be best approach to measure suppression of proliferation. We have removed this data and, as discussed above, performed a CFSE dilution assay to confirm differences in susceptibility to Treg-mediated suppression in NFAT1-competent and NFAT1-deficient T cells (Fig. 1F)

The 1998 Thornton and Shevach JEM paper describing the original in vitro suppression assay showed that anti-CTLA4 antibody had no effect on the ability of Treg cells to suppress. However, Figure 3E shows that CTLA4 inhibition restores full IL2 production by Th1 clones. Is there a disconnect between IL2 production and proliferation, or is this a difference between using Th1 clones and naïve CD4+ T cells in the suppression assay?

There has been some controversy regarding the role of Treg-expressed CTLA-4 in the regulation of Treg-mediated suppression. While the manuscript cited by the reviewer appears to point that way, later studies using CTLA-4 deficient TCR-transgenic or blocking Fab-fragments have supported an important role for this molecule, and suggested it can act by inhibiting B7 expression in DCs [2-6]. In order to understand the effect we described and to determine if CTLA-4 could be an important player in our system we decided to test it in our suppression reactions. Our results sided with the data that supports CTLa-4 involvement, though it is likely that in different contexts the relative role of this molecule in Treg-suppression may vary as other mechanisms might take over.

Why are different results observed for iTreg cells?

Our data suggest that iTreg-mediated suppression is NAFT-independent but other than that we still do not know much about how iTregs suppressed T helper cells. We decided, thus, to focus this report on describing the process that accounts for tTreg-mediated suppression of CD4+ T cells, although we currently actively pursuing the identification of the mechanisms that are activated in T cells when suppressed by iTregs.

In Figure 4B, the authors perform a conventional in vitro suppression assay. What happens in this assay to Nfat1-/- CD4+ T cells?

New data in Fig. 1F shows now that similar results are obtained when NFAT1-deficient naïve T cells are analyzed.

It seems that previous publications have described abnormal CD4+ T cell development in in DN-Ikaros mice. To show that the effects seen in Figure 4 are not due to developmental alterations, can the authors retrovirally express DN-Ikaros in WT CD4+ T cells, similar to expression of Nfat1 in Nfat1-/- cells in Figure 1?

We apologized for creating this confusion by not making this point it clearer in our original manuscript. We were also concerned about that fact and we performed the experiments suggested by the reviewer. We realized now that the fact that the data shown on Fig. 4A was obtained in T cells transiently transfected with a plasmid expressing a dominant negative Ikaros was only mention in the legend but not in the main text. This has been corrected now.

Also, the authors seem to be suggesting that Ikaros may mediate epigenetic changes at the IL2 locus. However, Figure 3 demonstrates that IL2 inhibition is not stable and that suppressed CD4+ T cells that have been rested for 4 hours reacquire the ability to make IL2.

We agree with this reviewer that this was a very interesting topic as it could explain the differences we saw in the stability of the anergic phenotype compared with that of suppressed cells. Our new data (Fig. 2F) shows that Tregs fail to induce a significant reduction in histone acetylation at the IL2 promoter. This could account for the fact that the effect of Ikaros on suppressed cells is transient as it does not lead to stable epigenetic

modifications. It is possible that other chromatin-remodeling proteins are also activated in anergic cells to cooperate with Ikaros in closing the IL2 locus and that these mechanisms are not fully activated in suppressed T cells. We are now continuing this line of research to understand better the exact epigenetic mechanisms that may explain the differences between the long-lasting inhibition of Il2 expression in anergic and the less stable inhibition in suppressed cells.

Referee #3:

We thank the reviewer for this comments and we have tried to address the concerns that were raised in the original manuscript to strengthen our conclusions.

1. Figure 1: it is not clear why the authors move between two different OVA-based readout systems, when the vast majority of the work is based in the DO11.10 system. This is confusing, particularly when different presentation styles are used for data analysis. For example in Figure 1A and B, both are supposed to be readouts for IL-2 levels using ELISA, yet where (A) gives no indication of ratios of Treg:Effectors and uses ng/ml concentrations for readouts (B) uses titrations and a relative IL-2 production levels. Consistency in readouts would enhance this figure.

We wanted to make sure that the dependency we saw on NFAT for Treg-mediated suppression was not strain-specific and we decided to analyze it in B6 and BalbC backgrounds. However, while some general aspects of this hypothesis are were analyzed in both strains (Fig. 1A and B for NFAT-dependency; Fig. 2B and C for ability of suppressed cells to activate NFAT) in other cases due in many cases to availability of specific reagents (clonotypic anti-TCR antibodies, transgenic IK-DN mice, congenic markers..) one or the other strain was chosen to further investigate a specific issue. We agreed with the reviewer that this may appear confusing but following his/her suggestions and to make results clearer we have now included this statement in the paper, change readouts to make them more similar (e.g. data Fig. 1A and B are now provided in absolute ng/ml) and indicate for every experiment the Tconv:Treg ratio used.

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Figure 1F. The figure legend does not make it clear how this assay was performed. Since both the effectors and Tregs transferred are CD4+ cells, how did the authors distinguish between the two. Potentially, transferred Treg cells could have lost Foxp3 expression and thus Tregs themselves could be incorporated in the readout. Similarly, not all Foxp3+ Treg cells are CD25+. This experiment needs performed using congenic mice and the absolute numbers of recovered effector CD4+ T cells need to be measured, not frequency.

This a very valid concerns and to address them we have now repeated these experiments shown in the now new Fig. 1G using congenic markers. Experiments were carried out by transferring Th1.2 naïve T cells and Thy1.1 Treg cells into Thy1.2 Rag1-/- hosts. Transferred donor T cells are now identified as CD4+CD3+Thy1.1-. Absolute numbers of cells are now also provided

Figure 1G, the rationale for this experiment- that NFAT1-deficiency may influence thymic development or differentiation of T helper cells- is confusing as the previous figures clearly show NFAT1-deficient T helper cells can produce IL-2 when appropriately stimulated. This experiment would be sufficiently pre-fixed by simply stating 'to confirm deficiency in NFAT1 contributes to inability of T helper cells to be suppressed by Tregs, we transduced....'.

We thank the reviewer for this comment. The statement has changed to indicate exactly that point.

Figure 2; again the use of two different OVA-based systems leads to confusion. In 2A, since GRAIL was not to be followed up, it may be best to leave that data out (not shown)

and focus on IKAROS. Although it may seem a minor point, swapping Fig 2F with 2E would make the data flow better and emphasis an interesting finding that although IL-2 suppression still occurred with iTregs present, IKAROS upregulation was impaired.

As suggested by the reviewer we have removed the data for Grail and swap Figs. 2F and E (now H and I) to create a better flow.

Fig 2G was very confusing. Normally effector cells are labelled with CFSE, transferred into mice with Treg cells (of another congenic strain) and then CFSE-dilution is monitored only in effector cells. In this case it is very difficult to tell what cells the BrdU is being assessed in as the gating system in not transparent. Also there seems to be little evidence that the effector cells were suppressed to any extent based on the FACS plots shown.

This point was also raised by referee#2 and therefore we decided to remove this data and instead include a CFSE dilution assay as suggested by the reviewer to show that NFAT1-deficient T cells are more resistant to Treg-mediated suppression that their wildtype counterparts.

Fig 3A and B, both figures were challenging to interpret due to their small size. Figures D-G add nothing to the manuscript as the data presented only reproduces well known aspects of potential Treg suppression mechanisms and since this manuscript provides no evidence that there is a direct link between NFAT1, IKAROS and loss of co-stimulation, then the authors should consider removing this data. This would free up space to expand the histological figures thereby enable better clarity for interpretation. In this section, abstract and introduction, the authors go into detail that NFAT1 acts independent of AP-1 to control effector cells via Treg cell suppression. However, not one experiment is conducted that looks at AP-1. These comments therefore need removed.

We apologize for the original size of the panels. We have re-structured this figure to increase the size of the IF panels but we still think that keeping figures D and G, while showing expected data, help strengthen our model (while address some of the concerns of referee #2). We have now rewritten some of the paragraphs to indicate that aspect. We had previously shown that Ikaros expression was dependent on NFAT but independent on AP-1. In fact co-stimulation prevented the formation of the NFAT-containing transcriptional complexes that direct the expression of Ikaros and other genes, likely by competition of those complexes by the high affinity NFAT/AP-1 complex [1, 7-9]. With the experiments shown in Fig. 3, we wanted to show that in suppressed T cells we could find a similar situation, where co-stimulation was reduced (Fig. 3D-E) (as in the conditions that induce anergy) and NFAT was activated in the absence of AP1 (Fig 3B and C). We have also included a new data shown that Ikaros expression is induced in T cells response to calcium signaling but blocked when other co-stimulatory pathways are activated with PMA (Fig. 3A).

Minor points:

Abstract, 4th line, the word 'by' is missing.
Thanks. It has been corrected.

Introduction, page 3, 6 line down, this sentence would read better as derived Treg cells in the thymus (tTregs).
Thanks. It has been corrected.

Results/discussion page 8, 8th line from bottom, suppressed should be suppress.
Thanks. It has been corrected

Page 9, first paragraph. Since the data is not being shown, the authors should state that here.

Thanks. It has been added.

Page 10, Fig 4C is actually Fig 3C.
Thanks. It has been corrected

Page 13, this reviewer does not think the data presented warrants the rather strong statement of 'this detailed understanding'. The data is nice, but far from detailed. This comment needs toning down.
This statement has been removed

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2nd Editorial Decision 05 June 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, referees 1 and 3 are now positive about its publication in EMBO reports. Referee 2 still has some concerns, which you might want to discuss in the final revision of your study. Although this would not be strictly necessary, other readers of your study might have similar doubts. On balance, however, I think your study is now suitable for publication in EMBO reports and I am thus writing with an 'accept in principle' decision. This means that I will be happy to officially accept your manuscript for publication, once a few minor issues/corrections have been dealt with, as follows.

- The legend to figure 4C is missing information about the number of mice analyzed in each group. In addition, the colors in the key do not seem to fully match the colors in the graph in this panel.

- As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. I have also included a bit more of the actual results you show in the abstract. Please find the edited versions below and let me know if you do NOT agree with any of the changes.

- We now encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet

or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports and congratulations on a successful publication!

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Edited title and abstract

Regulatory T cells suppress CD4+ T cells through NFAT-dependent transcriptional mechanisms

Regulatory T cells (Tregs) control autoreactive T cells by inhibiting activation-induced proliferation and cytokine expression. The molecular mechanisms responsible for the inactivation of effector T cells by Tregs remain yet to be fully characterized. We report that T-helper cells stimulated in the presence of Tregs quickly activate NFAT1 and have increased NFAT1-dependent expression of the transcription repressor Ikaros. NFAT1 deficiency or dominant negative Ikaros compromises Treg-mediated inhibition of T helper cells in vitro and in vivo. Thus, our results place NFAT-dependent mechanisms as general regulators of T cell tolerance and show that Treg-mediated suppression of T helper cells results from the activation of NFAT-regulated gene expression.
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REFEREE REPORTS

Referee #1:

The authors have largely addressed the previous concerns and the manuscript can be published in EMBO reports.

Referee #2:

The authors argued that interpretation of the experiments using mixed bone marrow chimeras would be complicated because of a problem of discriminating between the anergy induction in T cells and impaired Treg mediated suppression. The same problem applies to all experiments shown in the paper unless NFAT1 is deleted acutely.

Referee #3:

The authors have made a concerted effort to professionally tackle each of the comments made by all reviewers. The revised manuscript is improved, provides new information on the biology of Treg cells and will be of interest to a wide selection of readers.

2nd Revision - authors' response 16 June 2014

I am submitting a new version of our manuscript **EMBOR-2013-38233V2,** containing the changes you suggested. The corrected title and abstract are fine. I have also corrected the legend for Fig 4B and added the number of mice analyzed in Fig.4C. I have also submitted an excel data sheet with the raw data from all experiments and a file with the uncropped version of the gel shift shown in Fig 3B (it contains some extra lanes that we did not include in the figure but I have also labeled them just the same). I have also added two bullet points to the synopsis and created a graphic summary for the paper. Please let me know if there is any other thing you may need us to submit or change in the manuscript.

3rd Editorial Decision 16 June 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication.

