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LKB1 regulates TCR-mediated PLCγ1 activation and thymocyte positive selection

Yonghao Cao, Hai Li, Haifeng Liu, Min Zhang, Zichun Hua, Hongbin Ji, and Xiaolong Liu

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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.)

1st Editorial Decision 02 December 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the slight delay in getting back to you with a decision - this was primarily due to some difficulty in finding three appropriate referees. However, we have now received the comments from all three reviewers, which are enclosed below. As you will see, all three referees express interest in your finding that Lkb1 is involved in TCR signalling during thymocyte selection, but all also raise significant concerns that would need to be addressed by a major revision of your manuscript.

There are two major areas of criticism. Firstly, both referees 1 and 2 argue that you can not discriminate between a function for Lkb1 in regulating selection vs. a role in promoting survival of selected SP cells - which would be consistent with your previous work showing loss of Lkb1 leads to apoptosis of DP thymocytes. Referee 1 suggests ways to distinguish these possibilities (thymic reaggregation or fetal thymic culture experiments) and it will be critical that you address this central issue. Secondly, all three referees comment that further mechanistic insight into how Lkb1 fits into the TCR signalling cascade - specifically how it regulates PLCg1 activity, as well as demonstrating its regulation by Lck under physiologically relevant conditions. Again, further analysis and mechanistic insight on this point will be essential for eventual publication here.

In the light of the overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the

manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

MS# EMBOJ-2010-76341
"LKB1 regulates TCR-mediated PLCγ1 activation and thymocyte positive selection"
By Cao., et al

The authors are attempting to present new mechanistical evidence that the kinase, LKB1 contributes to regulating positive selection and survival of developing thymocytes by impinging on the intracellular signaling pathway regulated by the tyrosine kinase, Lck and the phospholipase, PLCγ1 . While they had previously documented a role for LKB1 in thymocyte ontogeny, they present further evidence that this kinase might be relevant in thymic selection by using TCR transgenic as well as LKB1 targeted mouse models in which LKB1 is specifically deleted starting at an early, DN stage of T cell differentiation (controlled by Lck-Cre) or a later stage (in DP via CD4-Cre). They found that LKB1 impairs differentiation of CD4CD8 DP thymocytes into functional SP cells probably by altering signal transduction at the level of PLCγ1 activity. While this study is of great interest to the field of T cell development, many of the conclusions made by the authors are either at odds with the presented results or could be interpreted in different ways.

As such, the authors claim a role for LKB1 in positive selection of DP thymocytes, a mechanism that ensures survival of self-tolerant, but functional T cells, while self-reactive cells are eliminated from the repertoire. The authors make their conclusion based mainly on the reduced relative numbers of SP cells found in LKB1 deficient mice, as well as a higher expression of the surface molecule HAS on LKB1KO SP cells (Figure 1). While these observations are sound, they are not sufficient to make LKB1 relevant for thymic selection. This reviewer feels very strongly that the authors should undertake thymic reagregation or fetal thymic cuture experiments to backup their claim of LKB1 playing a role in positive selection. This is feasible as they already have LKB1 deficiency established on the AND TCR background.

The authors have previously shown that LKB1 promotes survival of DP cells in response to energy depletion, such that increased thymocyte apoptosis is observed in absence of this kinase (Cao, Y. et al 2010, Cell Res and supplementary figure 4). The authors should consider the possibility that a similar mechanism may take place in normally selected SP, such that the reduced relative numbers of these cells is not due to defective positive selection, but due to an intrinsic metabolic defect of SP cells that would greatly impair the completion of their maturation cycle such as reduced downregulation of HAS. It is noteworthy that HAS downregulation from the surface of LKB1-deficient AND TCR SP cells (figure 1D) is almost identical to their LKB1 littermates, yet there is a ~4 fold reduction in the % of LKB1-deficient AND CD4 SP cells (figure 1C). This would be at odds with the authors' claim that "LKB1 regulates positive selection". However, this could be explained

by distinct energy stress levels in TCR transgenic and non-transgenic animals, as VDJ recombination does not take place in the former leaving enough energy (ATP) for downregulating HAS. This, of course is assuming the TCR transgenics are on a Rag deficient background which, even though not explicitly mentioned in "Materials & Methods" is inferred by the absence of CD8 cells in supplementary figure 2. It is noteworthy to the authors that this figure is very confusing to read as panels A and B are labeled for 2 distinct Cre promoters driving LKB1 deletion. The authors may want to carefully edit figures as this reviewer found a number of errors throughout the manuscript. Similarly, Figure 3 as a whole needs editing and this reviewer would suggest to adding it in the supplementary material. Indeed, while the authors have gone trough a great deal of work by generating a second conditional LKB1 mouse model (targeted by CD4-Cre at the DP stage), the gathered info are redundant with the previous mouse model in which LKB1 is deleted under the control of Lck-Cre. It is specially overstated at best, that having reduced numbers of SP after deleting LKB1 at the DP stage is proof of the relevance of this kinase for positive selection. This reviewer reiterates the concerns made above as far as the authors' conclusions concerning LKB1 and positive selection. Delaying the abrogation LKB1 further down the road of thymocyte development is not proof per se that LKB1 regulates positive selection.

Also, this reviewer disagrees with the authors' claim of lower expression of CD5 on the surface of LKB1-deficient SP cells (figure 2C, OR 3G). Both levels (wild type and LKB1 KO) seem rather similar. Maybe presenting a statistical analysis would be more appropriate. A similar comment is made for the TCR expression of figure 3F. Considering TCR levels to be equal on wt and LKB1KO cells would substantiate the authors claim of intracellular differences. If instead, fewer TCR high cells are present in LKB1-deficient thymocytes as claimed by the authors (figure 3F) , the subsequent reduction of intracellular signaling would be a logical consequence thereof. This would be very pertinent for the Ca2+ spick of Figure 4D. Indeed, there is no delay in release of the ion Calcium, which one would expect if a major defect in proximal signaling had occurred, instead a shorter amplitude of the Ca2+ flux in LKB1KO thymocytes is reported by the authors. The type of Ca transient presented is more reminiscent of Ca flux observed following thymocyte stimulation with high affinity ligands that induce negative selection as opposed to the Ca transient one would observe during positive selection. Therefore, the authors' conclusions about the presented molecular mechanism whereby LKB1 would facilitate signaling resulting in thymocyte positive selection and differentiation are not substantiated by their results.

Similarly, there is quite some discrepancy between the authors' conclusions and their presented data of figure 4B. This reviewer strongly objects to the authors' claim that only PLCγ1 phosphorylation is diminished in LKB1-deficient thymocytes, but not src or ZAP 70. It seems that except of the phosphorylation of LAT, all other presented signals are diminished in absence of LKB1 at a 1 min stimulation time point (how about ERK phosphorylation ?). A number of publications have documented a strong and transient signaling kinetics leads to negative selection, while a slow and sustained signaling controls thymocyte differentiation. To this reviewer, the data presented would rather imply a lack of the former than a kinetic defect of the latter, indicating that if ever LKB1 would be more relevant to negative than positive selection !!!

Last but not least it is noteworthy that the tyrosine phosphorylation kinetics of LKB1 (figure5A) are very much at odds with the kinetics of PLCγ1 phosphorylation presented in figure 4A. Despite this discrepancy the authors conclude from the remaining data that the phosphorylation of at least 3 tyrosine residues of LKB1 by Lck are required for not only maximal binding of PLCγ1 to LAT, but also maximal phosphorylation of the phospholipase. This reviewer is wondering how can the delayed phosphorylation of LKB1 possibly contribute in regulating the previously occurring phosphorylation of PLCγ1?

Overall, this work is certainly interesting but the results are not always supporting the claims or at least alternate conclusions can, be made. This reviewer feels that this manuscript is premature for publication in its current form and major revision should be made before it could again be considered for publication in EMBO J.

Referee #2:

In the current study, Cao et al. reported that the serine/threonine kinase LKB1 was involved in the positive selection of thymocytes through regulation of TCR signaling. LKB1 was directly phosphorylated by Lck at tyrosine residues 36, 261 and 365 and preferentially interacted with LAT and PLCg1 in a phosphorylation-dependent manner. Loss of LKB1 impaired recruitment of PLCg1 to the LAT signalosome following TCR stimulation, attenuated tyrosine phosphorylation of PLCg1, eventually resulting in impaired TCR signal transduction. Thus, the authors concluded that LKB1 is a critical component involved in TCR signaling. The concept that LKB1 is involved in the TCR signaling is novel and potentially coordinates increased metabolism and TCR signaling. My specific comments are as follow:

Major points:

1. The authors tried to demonstrate that LKB1 was phosphorylated by Lck at tyrosine residues 36, 261 and 365 in TCR signal transduction. However, the study was performed either in the presence of an Lck inhibitor or in 293T when proteins were overexpressed. Studies performed in a T cell line or in primary T cells are necessary to demonstrate whether these tyrosin residues are phosphorylated by Lck after TCR engagement. More functional readout, in addition to the PLCg1 phosphorylation analysis (fig 6E), should be provided to demonstrate that phosphorylation on these tyrosine residues has biological significance in TCR signal transduction.

2. The authors showed in DP thymocytes that LKB1 deficiency impaired TCR-mediated PLCg1 phosphorylation and calcium mobilization, both of which could be the consequences of defective PLCg1 recruitment by LAT. Demonstrating the mechanism by which LKB1 regulates PLCg1 and LAT interaction would substantially improve the quality of the manuscript.

3. The impaired development of SP thymocytes in the presence of LKB1 deficiency could be the result of failed thymocyte amplification and/or survival following positive selection, not necessarily defective positive selection per se. Therefore it is not proper to include positive selection in the title. The author should elaborate this point in the discussion or distinguish these two possibilities if possible.

Minor points:

1. Figure legends should provide sufficient information to explain the experiments. For example, in fig 4A, it is important to state how long the cells were stimulated given that thymocytes are prone to apoptosis in culture. In this regard, the percentage of viable thymocytes should also be examined,

2. No data was provided to support the statement that "LKB1 preferentially interacted with LAT in a phosphorylation-dependent manner" (Abstract)

Referee #3:

Previously, the authors and the Cantrell group have shown that the serine/threonine kinase LKB1 is critical for T cell development. In this manuscript, Cao and coworkers examine whether this kinase is critical for the positive selection of single positive T cells and uncover a potential mechanism for these effects. The authors found that LKB1 was required for positive selection and the production of single positive peripheral T cells. Intriguingly, the authors observe that LKB1 is tyrosine phosphorylated upon TCR induction and the presence of LKB1 is critical for the function, phosphorylation and LAT binding capacity of PLCγ1. The role of LKB1 in T cell development and signaling has only recently been appreciated and thus, insight into the function of this protein is needed. Importantly, the observation that LKB1 is needed for the function of PLCγ1 is especially novel and provides a new player in the formation of the LAT signalsome. However, there are several experiments that would strengthen the key findings of this manuscript.

1) In all the figures, the control mice are reported as LckCre-LKB1+/fl. However, the text reports the control mice as LckCre+LKB1+/fl. Both mice would be appropriate controls, but exactly which mice are reported/used in each experiment is needed.

2) In Figure 4B, the authors show the TCR-induced phosphorylation of Lck, ZAP-70, LAT and PLCγ1 in LKB1 sufficient and deficient mice. To better assess the effects of LKB1 on the

phosphorylation of these proteins, the extent of phosphorylation of each protein should be quantified by densitometry for each experiment. The values for the quantification for the three experiments should be averaged to provide measure of differences in phosphorylation across multiple experiments. In addition, the authors examine only the phosphorylation of LAT at tyrosine 175. However, the main point of the paper is that LKB1 is needed for the interaction of PLCγ1 with LAT. Thus, examining the effect of LKB1 on LAT tyrosine 136 phosphorylation is needed in order to determine if the effects of LKB1 on the PLCγ1/LAT interaction is due to alterations LAT phosphorylation at the direct PLCγ1 binding site.

3) Figure 5 clearly shows that Lck is capable of phosphorylating LKB1. However, it is less convincing that Lck is the direct in vivo kinase. The Lck inhibitor studies are especially unconvincing. The authors used a single unreported concentration of the Lck inhibitor. To conclusively show that the effects of the inhibitor on in vivo LKB1 phosphorylation are due to effects on Lck, the authors need to use a dose response of inhibitor and examine substrates downstream of both Lck and Fyn. In the end, either the conclusions must be weakened to take into account that Fyn may also play a role in the in vivo phosphorylation of LKB1 or the authors should examine the role Fyn may play in these events.

4) In Figure 7C, the authors show that LKB1 deletion results in decreased interaction of LAT with PLCγ1. Is the effect across the entire timecourse or localized only to the single early timepoint shown? Since this is a key finding of the paper, this experiment should be expanded beyond a single early timepoint. Additionally, is the effect of LKB1 on LAT ligand binding localized to PLCγ1? The binding of LAT to other ligands, such as Grb2, SLP-76 and/or GADS, should be examined to determine if this is a global suppression of LAT binding to multiple ligands.

1st Revision - Authors' Response 24 January 2011

Referee #1

The authors are attempting to present new mechanistical evidence that the kinase, LKB1 contributes to regulating positive selection and survival of developing thymocytes by impinging on the intracellular signaling pathway regulated by the tyrosine kinase, Lck and the phospholipase, PLCγ1. While they had previously documented a role for LKB1 in thymocyte ontogeny, they present further evidence that this kinase might be relevant in thymic selection by using TCR transgenic as well as LKB1 targeted mouse models in which LKB1 is specifically deleted starting at an early, DN stage of T cell differentiation (controlled by Lck-Cre) or a later stage (in DP via CD4-Cre). They found that LKB1 impairs differentiation of CD4CD8 DP thymocytes into functional SP cells probably by altering signal transduction at the level of PLCγ1 activity. While this study is of great interest to the field of T cell development, many of the conclusions made by the authors are either at odds with the presented results or could be interpreted in different ways.

We thank the referee for his/her interest in our study. Improvements have been made accordingly in the revised manuscript.

As such, the authors claim a role for LKB1 in positive selection of DP thymocytes, a mechanism that ensures survival of self-tolerant, but functional T cells, while self-reactive cells are eliminated from the repertoire. The authors make their conclusion based mainly on the reduced relative numbers of SP cells found in LKB1 deficient mice, as well as a higher expression of the surface molecule HAS on LKB1KO SP cells (Figure 1). While these observations are sound, they are not sufficient to make LKB1 relevant for thymic selection. This reviewer feels very strongly that the authors should undertake thymic reagregation or fetal thymic culture experiments to backup their claim of LKB1 playing a role in positive selection. This is feasible as they already have LKB1 deficiency established on the AND TCR background.

Thymocyte positive selection is accompanied by the upregulation of CD5, CD69 and TCR molecules (Azzam et al, 1998; Bendelac et al, 1992; Lucas et al, 1994; Starr et al, 2003; Swat et al, 1993), and induces two key events (the survival and the differentiation) (Alberola-Ila & Hernández-Hoyos, 2003; Bosselut, 2004; Kersh, 2004). In the original version, we had showed that LKB1-deficient thymocytes were unable to express CD5, CD69 and TCR molecules at a level as that of their counterparts and failed to upregulate Bcl-2 and IL-7R molecules (for survival). In this revised version, we added new data showing that LKB1-dificient DP thymocytes failed to upregulate the

lineage-specifying factors ThPOK and Runx3 (for differentiation). Taken together, we conclude that LKB1 regulates positive selection, and we think the decreased HSA^{low} SP cell population in LKB1-deficient mice is a consequence of impaired positive selection.

In addition, the attenuated TCR signaling in the absence of LKB1 supports our conclusion that LKB1 is involved in thymocyte positive selection.

We have done the FTOC according to the reviewer's suggestion. The results showed that AND TCR transgenic LKB1-deficient DP thymocytes failed to differentiate into CD4 SP (Figure 1F).

The authors have previously shown that LKB1 promotes survival of DP cells in response to energy depletion, such that increased thymocyte apoptosis is observed in absence of this kinase (Cao, Y. et al 2010, Cell Res and supplementary figure 4). The authors should consider the possibility that a similar mechanism may take place in normally selected SP, such that the reduced relative numbers of these cells is not due to defective positive selection, but due to an intrinsic metabolic defect of SP cells that would greatly impair the completion of their maturation cycle such as reduced down regulation of HSA.

We thank the reviewer for his/her nice point. We can't exclude that metabolic defect might also contribute to the impaired generation of SP thymocytes in LKB1-deficient mice. We have mentioned this issue in the Discussion part (page 16). In the revised version, we added new data showing that LKB1-deficient DP thymocytes failed to differentiate further into CD4 and CD8 SP cells even if their survival was prolonged by ectopic expression of the antiapoptotic Bcl-XL protein (Figure 7C and D).

It is noteworthy that HSA down regulation from the surface of LKB1-deficient AND TCR SP cells (figure 1D) is almost identical to their LKB1 littermates, yet there is a ~4 fold reduction in the % of LKB1-deficient AND CD4 SP cells (figure 1C). This would be at odds with the authors' claim that "LKB1 regulates positive selection". However, this could be explained by distinct energy stress levels in TCR transgenic and non-transgenic animals, as VDJ recombination does not take place in the former leaving enough energy (ATP) for down regulating HAS. This, of course is assuming the TCR transgenics are on a Rag deficient background which, even though not explicitly mentioned in "Materials & Methods" is inferred by the absence of CD8 cells in supplementary figure 2.

We noticed that the down regulation of surface HSA from DP to SP cells is not dramatic under TCR transgene background. We have remade the Figure (1D), in which the HSA expression level of AND TCR transgenic LKB1-deficient DP thymocytes was used as control. The new Figure 1D showed that the down regulation of surface HSA is impaired in the absence of LKB1.

The AND TCR transgene was not based on a Rag deficient background [this had been used in many other studies (Fischer et al, 2005; Fujimoto et al, 2002; Liu & Bosselut, 2004)], and $V\alpha11^{high}$ cells were gated for analysis as indicated in the manuscript.

It is noteworthy to the authors that this figure is very confusing to read as panels A and B are labeled for 2 distinct Cre promoters driving LKB1 deletion. The authors may want to carefully edit figures as this reviewer found a number of errors throughout the manuscript.

We apologize for the mistake. In panel B of Supplementary Figure 2, we intended to show the absolute CD4 T cell numbers in AND-TCR transgenic *Lck*Cre^{-}*LKB1*$^{+/fl}$ or *Lck*Cre^{+}*LKB1*$^{fl/fl}$ mice. We have corrected the labeling.

Other improvements have been made accordingly in the manuscript.

Similarly, Figure 3 as a whole needs editing and this reviewer would suggest to adding it in the supplementary material. Indeed, while the authors have gone trough a great deal of work by generating a second conditional LKB1 mouse model (targeted by CD4-Cre at the DP stage), the gathered info are redundant with the previous mouse model in which LKB1 is deleted under the control of Lck-Cre. It is specially overstated at best, that having reduced numbers of SP after deleting LKB1 at the DP stage is proof of the relevance of this kinase for positive selection. This reviewer reiterates the concerns made above as far as the authors' conclusions concerning LKB1 and positive selection. Delaying the abrogation LKB1 further down the road of thymocyte development is not proof per se that LKB1 regulates positive selection.

We had found a substantially developmental block at the DN3 to DN4 transition in Lck-Cre mediated LKB1-deficient mice (could be due to attenuated pre-TCR signaling), thus, we generated *Cd4*Cre^{+}*LKB1*$^{fl/fl}$ mice to exclude the possibility that the differences in thymocyte maturation between wild-type and LKB1-deficient mice resulted from changes arising before the DP stage and to further verify whether LKB1 is needed for the differentiation of DP thymocytes into CD4 and CD8 SP cells.

We have added this part as supplementary material according to the reviewer's suggestion in the revised version (Supplementary Figure 3).

Also, this reviewer disagrees with the authors' claim of lower expression of CD5 on the surface of LKB1-deficient SP cells (figure 2C, OR 3G). Both levels (wild type and LKB1 KO) seem rather similar. Maybe presenting a statistical analysis would be more appropriate. A similar comment is made for the TCR expression of figure 3F.

We mentioned that the transitional TCR^{high}CD4^{+}CD8^{low} LKB1-deficient cells did not fully upregulate CD5 or CD69 in the absence of LKB1. We agree with the reviewer that CD5 level is not dramatically changed and we have changed these statements in the revised version (page 7).

Considering TCR levels to be equal on wt and LKB1KO cells would substantiate the authors claim of intracellular differences. If instead, fewer TCR high cells are present in LKB1-deficient thymocytes as claimed by the authors (figure 3F), the subsequent reduction of intracellular signaling would be a logical consequence thereof. This would be very pertinent for the Ca2+ spick of Figure 4D.

There were fewer TCR^{high} DP cells presented in LKB1-deficient thymocytes, however, the dominant population of LKB1-deficient DP thymocytes (TCR^{int} DP cells) had the very similar surface expression level of TCR as their counterparts from control littermates (Figure 2A).

Indeed, there is no delay in release of the ion Calcium, which one would expect if a major defect in proximal signaling had occurred, instead a shorter amplitude of the Ca2+ flux in LKB1KO thymocytes is reported by the authors. The type of Ca transient presented is more reminiscent of Ca flux observed following thymocyte stimulation with high affinity ligands that induce negative selection as opposed to the Ca transient one would observe during positive selection. Therefore, the authors' conclusions about the presented molecular mechanism whereby LKB1 would facilitate signaling resulting in thymocyte positive selection and differentiation are not substantiated by their results.

We understand the reviewer's concerns. However, the relationship between Ca^{2+} Flux behaving and positive selection seems still inconclusive. The deletion of many factors involved in thymocyte positive selection showed no Ca^{2+} mobilization spick delay after T cell crosslinking (Albu et al, 2007; Fu et al, 2009; Sommers et al, 2005), though some of them did indeed (Maltzman et al, 2005; Swat et al, 2006). We also noticed the delayed Ca^{2+} mobilization spick was mainly found in CD4 SP cells, but not in DP thymocytes (Liu et al, 2003; Davalos-Misslitz et al, 2007).

Similarly, there is quite some discrepancy between the authors' conclusions and their presented data of figure 4B. This reviewer strongly objects to the authors' claim that only PLCγ1; phosphorylation is diminished in LKB1-deficient thymocytes, but not src or ZAP 70. It seems that except of the phosphorylation of LAT, all other presented signals are diminished in absence of LKB1 at a 1 min stimulation time point (how about ERK phosphorylation?). A number of publications have documented a strong and transient signaling kinetics leads to negative selection, while a slow and sustained signaling controls thymocyte differentiation. To this reviewer, the data presented would rather imply a lack of the former than a kinetic defect of the latter, indicating that if ever LKB1 would be more relevant to negative than positive selection !!!

We have quantified the images and the results showed that only PLCγ1 phosphorylation was significantly impaired when LKB1 was ablated (Supplementary Figure 4).

The phosphorylation of ERK was attenuated in the absence of LKB1 (Figure 1).

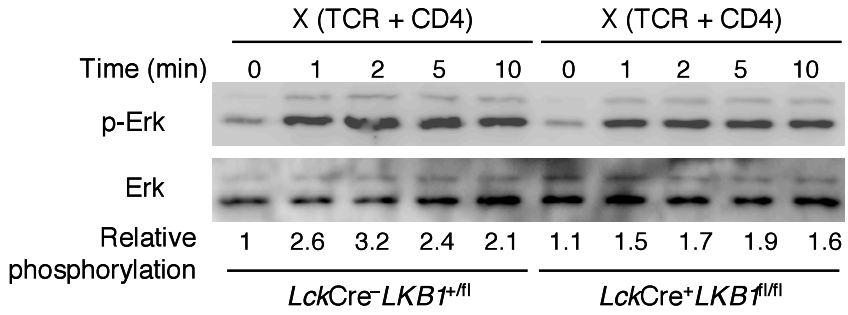

Figure 1 The phosphorylation of ERK in the absence of LKB1. Sorted DP thymocytes from *Lck*Cre^{+}*LKB1*$^{fl/fl}$ mice or littermate controls were labeled with biotinylated anti-TCR and anti-CD4 and were then cross-linked with streptavidin for the indicated times or left untreated (0 min). Total lysates from these cells were subjected to SDS-PAGE and analyzed with antibodies against the phosphorylation of Erk. The expression of total Erk served as a loading control. The numbers underneath the bands refer to the relative phosphorylation level.

We understand the review's concerns that LKB1 may be also relevant to negative selection. We didn't investigate the role of LKB1 in negative selection in this study and we may try later.

Last but not least it is noteworthy that the tyrosine phosphorylation kinetics of LKB1 (figure5A) are very much at odds with the kinetics of PLCγ1; phosphorylation presented in figure 4A. Despite this discrepancy the authors conclude from the remaining data that the phosphorylation of at least 3 tyrosine residues of LKB1 by Lck are required for not only maximal binding of PLCγ1; to LAT, but also maximal phosphorylation of the phospholipase. This reviewer is wondering how can the delayed phosphorylation of LKB1 possibly contribute in regulating the previously occurring phosphorylation of PLCγ1?

This is a legitimate concern and we thank the reviewer for pointing this out. These experiments (Figure 4B and Figure 5A in the original version, Figure 3B and Figure 4A in the revised version) were not done parallelly, there might be some variation on the time interval. In fact, we had found LKB1 was phosphorylated within 1 min after anti-TCR and anti-CD4 crosslinking in many other experiments. Accordingly, the association between LKB1 and LAT or between LKB1 and PLCγ1 at 1 min was enhanced after TCR stimulation (Figure 6A and C). We have replaced the figure in the revised version (Figure 4A).

Overall, this work is certainly interesting but the results are not always supporting the claims or at least alternate conclusions can, be made. This reviewer feels that this manuscript is premature for publication in its current form and major revision should be made before it could again be considered for publication in EMBO J.

We thank the reviewer for his/her criticism and great suggestions, all these have significantly improved our manuscript.

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Referee #2:

In the current study, Cao et al. reported that the serine/threonine kinase LKB1 was involved in the positive selection of thymocytes through regulation of TCR signaling. LKB1 was directly phosphorylated by Lck at tyrosine residues 36, 261 and 365 and preferentially interacted with LAT and PLCg1 in a phosphorylation-dependent manner. Loss of LKB1 impaired recruitment of PLCg1 to the LAT signalosome following TCR stimulation, attenuated tyrosine phosphorylation of PLCg1, eventually resulting in impaired TCR signal transduction. Thus, the authors concluded that LKB1 is a critical component involved in TCR signaling. The concept that LKB1 is involved in the TCR signaling is novel and potentially coordinates increased metabolism and TCR signaling.

We thank the referee for these positive comments.

My specific comments are as follow:

Major points:

1. The authors tried to demonstrate that LKB1 was phosphorylated by Lck at tyrosine residues 36, 261 and 365 in TCR signal transduction. However, the study was performed either in the presence of an Lck inhibitor or in 293T when proteins were overexpressed. Studies performed in a T cell line or in primary T cells are necessary to demonstrate whether these tyrosin residues are phosphorylated by Lck after TCR engagement. More functional readout, in addition to the PLCg1 phosphorylation analysis (fig 6E), should be provided to demonstrate that phosphorylation on these tyrosine residues has biological significance in TCR signal transduction.

We agree with the referee's concerns. We have addressed this issue by using the Jurkat T cell line. All the single mutants and the double mutants attenuated LKB1 tyrosine phosphorylation following TCR stimulation. And the dramatic decrease in tyrosine phosphorylation of the triple mutant suggested that these residues represented the major tyrosine phosphorylation sites of LKB1 in response to TCR ligation. This result has been added in the revised version (Figure 5D, Page 11).

We have showed that the phosphorylation of these three tyrosine promoted the association between LKB1 and PLCγ1 (Figure 6F) and was required for the PLCγ1 phosphorylation upon TCR stimulation (Figure 5F). To investigate more functional readout regarding to these three residues is a very attractive suggestion. And making the knocking mice with LKB1 single-mutant (Tyr36) and double-mutant (Tyr261 and Tyr365) are in process.

2. The authors showed in DP thymocytes that LKB1 deficiency impaired TCR-mediated PLCg1 phosphorylation and calcium mobilization, both of which could be the consequences of defective PLCg1 recruitment by LAT. Demonstrating the mechanism by which LKB1 regulates PLCg1 and LAT interaction would substantially improve the quality of the manuscript.

In the original version we have demonstrated that LKB1 regulates PLCγ1 and LAT interaction by co-IP and western blot analysis. In the revised version, we showed that LKB1 promotes the recruitment of PLCγ1 to the LAT signalosome by confocal experiments (Figure 6B and E).

3. The impaired development of SP thymocytes in the presence of LKB1 deficiency could be the result of failed thymocyte amplification and/or survival following positive selection, not necessarily defective positive selection per se. Therefore it is not proper to include positive selection in the title. The author should elaborate this point in the discussion or distinguish these two possibilities if possible.

We understand the reviewer's concerns that other possibilities may also contribute to the impaired generation of SP thymocytes. As we mentioned above, thymocyte positive selection is accompanied by the upregulation of CD5, CD69 and TCR molecules (Azzam et al, 1998; Bendelac et al, 1992; Lucas et al, 1994; Starr et al, 2003; Swat et al, 1993), and induces two key events (the survival and the differentiation) (Alberola-Ila & Hernández-Hoyos, 2003; Bosselut, 2004; Kersh, 2004). In the original version, we had showed that LKB1-deficient thymocytes were unable to express CD5, CD69 and TCR molecules at a level as that of their counterparts and failed to upregulate Bcl-2 and IL-7R molecules (for survival). In this revised version, we added new data showing that LKB1-dificient DP thymocytes failed to upregulate the lineage-specifying factors ThPOK and Runx3 (for differentiation). We think the decreased HSA^{low} SP cell population is a consequence of impaired positive selection. These finding taken together suggested that the initiation, the progression and the completion of positive selection were impaired in the absence of LKB1. Therefore, we prefer to keep "positive selection". Alternatively, we have discussed the other possibilities in the Discussion part (Page 16).

We have tried to distinguish thymocyte survival and positive selection by using FTOC experiment. LKB1-deficient DP thymocytes failed to differentiate further into CD4 and CD8 SP cells even if their survival was prolonged by ectopic expression of the antiapoptotic Bcl-XL protein (Figure 7C and D).

Minor points:

1. Figure legends should provide sufficient information to explain the experiments. For example, in fig 4A, it is important to state how long the cells were stimulated given that thymocytes are prone to apoptosis in culture. In this regard, the percentage of viable thymocytes should also be examined,

Thymoyctes were stimulated for 16 h for analysis. The percentage of viable thymocytes is about 40% for control thymocytes and 30% for LKB1-dificient cells. We gated on live cells for all these analysis.

Other improvements have been made accordingly in the manuscript as highlighted.

2. No data was provided to support the statement that "LKB1 preferentially interacted with LAT in a phosphorylation-dependent manner" (Abstract)

We have changed this sentence (page 2).

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Referee #3:

Previously, the authors and the Cantrell group have shown that the serine/threonine kinase LKB1 is critical for T cell development. In this manuscript, Cao and co-workers examine whether this kinase is critical for the positive selection of single positive T cells and uncover a potential mechanism for these effects. The authors found that LKB1 was required for positive selection and the production of single positive peripheral T cells. Intriguingly, the authors observe that LKB1 is tyrosine phosphorylated upon TCR induction and the presence of LKB1 is critical for the function, phosphorylation and LAT binding capacity of PLCγ1. The role of LKB1 in T cell development and signaling has only recently been appreciated and thus, insight into the function of this protein is needed. Importantly, the observation that LKB1 is needed for the function of PLCγ1 is especially novel and provides a new player in the formation of the LAT signalsome.

We thank the referee for these positive comments.

However, there are several experiments that would strengthen the key findings of this manuscript.

1) In all the figures, the control mice are reported as LckCre-LKB1+/fl. However, the text reports the control mice as LckCre+LKB1+/fl. Both mice would be appropriate controls, but exactly which mice are reported/used in each experiment is needed.

LckCre^-*LKB1*$^{+/fl}$ mice were used as controls in all experiments. In the Supplementary Figure 3 of the original version, we compared the expression levels of CD5, CD69 and TCRβ on DP thymocytes from *Lck*Cre^-*LKB1*$^{+/fl}$ and *Lck*Cre^+*LKB1*$^{+/fl}$ mice in order to show that *Lck*Cre itself has no impact on the expression of these molecules. We have deleted this Supplementary Figure in the revised version considering it is somewhat confusing.

2) In Figure 4B, the authors show the TCR-induced phosphorylation of Lck, ZAP-70, LAT and PLCγ1 in LKB1 sufficient and deficient mice. To better assess the effects of LKB1 on the phosphorylation of these proteins, the extent of phosphorylation of each protein should be quantified by densitometry for each experiment. The values for the quantification for the three experiments should be averaged to provide measure of differences in phosphorylation across multiple experiments. In addition, the authors examine only the phosphorylation of LAT at tyrosine 175. However, the main point of the paper is that LKB1 is needed for the interaction of PLCγ1 with LAT. Thus, examining the effect of LKB1 on LAT tyrosine 136 phosphorylation is needed in order to determine if the effects of LKB1 on the PLC-γ1/LAT interaction is due to alterations LAT phosphorylation at the direct PLCγ1 binding site.

The results of image quantification were added as Supplementary Figure 4.

We have examined the LAT tyrosine 136 phosphorylation according to the reviewer's suggestion and the results showed that LKB1 deficiency has no detectable impact on the phosphorylation of LAT tyrosine 136. Given the similar results, we have replaced the anti-phospho-LAT Tyr175 with anti-phospho-LAT Tyr136 in the revised version (Page 19 and Figure 3B).

3) Figure 5 clearly shows that Lck is capable of phosphorylating LKB1. However, it is less convincing that Lck is the direct in vivo kinase. The Lck inhibitor studies are especially unconvincing. The authors used a single unreported concentration of the Lck inhibitor. To conclusively show that the effects of the inhibitor on in vivo LKB1 phosphorylation are due to effects on Lck, the authors need to use a dose response of inhibitor and examine substrates downstream of both Lck and Fyn. In the end, either the conclusions must be weakened to take into account that Fyn may also play a role in the in vivo phosphorylation of LKB1 or the authors should examine the role Fyn may play in these events.

We had done dose-dependent experiments regarding to the Lck inhibitor and have added this results as Supplementary Figure 5 in the revised version. This inhibitor has been well defined as an Lck II specific inhibitor and widely used by others (Burchat et al, 2000; Treanor et al, 2006).

Lck is the main player of Src family in T cell, although Fyn could partially compensate for Lck. Interestingly, Fyn might not be able to phosphorylate LKB1 at tyrosine residues 36 (new data, Supplementary Figure 7). Given the phosphorylation of LKB1 Tyr36 is needed for fully activation of PLCγ1 (Fig 5E and F), Fyn might not be able to play an equivalent role as Lck in this process.

4) In Figure 7C, the authors show that LKB1 deletion results in decreased interaction of LAT with PLCγ1. Is the effect across the entire timecourse or localized only to the single early timepoint shown? Since this is a key finding of the paper, this experiment should be expanded beyond a single early timepoint. Additionally, is the effect of LKB1 on LAT ligand binding localized to PLCγ1? The binding of LAT to other ligands, such as Grb2, SLP-76 and/or GADS, should be examined to determine if this is a global suppression of LAT binding to multiple ligands.

We understand the reviewer's concerns. The experiments were repeated at different time courses according to his/her suggestion.

Since a commercial GADS antibody was currently unavailable, we examined the binding of LAT to Grb2 and SLP-76. And the results showed that the binding of LAT to PLCγ1, but not to either Grb2 or SLP-76, was attenuated upon TCR stimulation in the absence LKB1 (Figure 5D).

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Additional Editorial Correspondence 21 February 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-76341R to the EMBO Journal. It has now been seen again by all three referees, whose comments are enclosed below. As you will see, referees 2 and 3 are overall happy with the revision, although referee 2 does still have some minor remaining issues. Referee 1, however, raises serious concerns - primarily with the FTOC experiments - and is therefore unable to recommend publication of your study.

His/her main issue is that you do not appear to have used exogenous selection ligands in your FTOC experiments, and therefore the results from these analyses are inconclusive. Consequently, he/she still argues that your data do not distinguish between a role for LKB1 in positive selection vs. a role in survival of SP cells.

This is clearly a critical issue, and before taking a decision on your manuscript, I think it would be very helpful if you could provide me with a response to the concerns raised by this reviewer. Most importantly, I need to know whether you did use ligands in your FTOC experiments, but a complete point-by-point response to the review would be valuable - so that I can, if necessary, consult further with this referee.

I look forward to your response.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee 1:

Since the first submission, the authors have undertaken a significant amount of work to improve the quality of the manuscript by attempting to answer the reviewers concerns. Unfortunately, they main findings as to the role of LKB1 in promoting positive selection are still inconclusive or overstated at best and inaccurate at worst. That LKB1 regulates positive selection is one of the main claims of this work as indicated in the title and throughout the manuscript and should therefore be accurately documented. Thus, the FTOC generated in response to the reviewer's request (Figure 1f) does not prove a defect in selection per se, as the authors did not add exogenous selection ligands (at least nothing the like is mentioned in the figure legends or the methods section). Indeed as the fetal circulatory system is not providing ligands anymore and as endogenous thymic ligands are exhausted after a couple of days, one has to add ligands for the transgenic TCR to the culture in order to observe true positive or negative selection.

(Another accepted method in the field of T-cell ontogeny is to test thymic selection by generating H-Y TCR transgenics on the deficient background of the gene of interest. As the H-Y TCR recognizes a male antigen one can test thymic selection based the presence or absence of T cells in male or female pups).

Having less SPs in the LKb1 FTOCs in absence of ligand as the authors observe would be more indicative of increased cell death, especially after 6 days of culture (is the same difference observed at day 0?). This is in fact supported by figure 7c in which a higher proportion of Annexin V stained cells (22.4%) is observed in LKB1-defective FTOCs as opposed to wt cultures (15%). Augmented cell death would actually be more inline with the authors previous observation that increased apoptosis is observed in absence of LKB1. It would thus be possible that Lkb1 is required to promote a post-selection survival signal to differentiated thymocytes as has been described for NFkB.

A similarly concern as for the conclusions of figure 1F is raised for Figure 7D, ectopically expressed Bcl-X cannot possibly increase the proportion of SP because there is no selection ligand added. Since thymocyte's positive (differentiation) or negative selection (death) is resulting from active

TCR engagement, only a post-selection stage would be influenced by transfected Bcl-X, but not the selection process itself in absence of ligand. This is supported by the fact that Bcl-X expression does not change either the proportion of subsets in the wild-type FTOCs. If Bcl-X affected thymic selection one would expect to see a change in the latter.

As the FTOCs experiments are widely inconclusive, the authors have so far only shown marginal expression differences of surface receptors CD5, CD69, TCR, CD24 to support their claim of relevance of LKb1 in thymic selection. This is by far to little, especially as the authors have not considered entertaining the sound comment of reviewer 1 as to "include statistical analysis" to better support expression differences of the analyzed receptors. Instead, the authors have introduced FACS measurements of IL-7R and BCL-2, two receptors that are required for survival of differentiated SP cells. Again even though decreased expression of those 2 receptors is measured in absence of LKB1, it is not proof of a bona fide defect in positive selection. Indeed, the authors in their rebuttal (bottom page 1) agree to a multi-phase process of transition from DP to SP ('the survival and the differentiation). What they fail to mention is that there is in fact at least 4 distinct phases (negative and positive selection, lineage commitment as well as survival). The TCR engagement characterizes positive and negative selection, distinct gene expression such as ThPOK or Runx3 accompanies lineage commitment and upregulation of survival markers such as IL-7R denote the last phase.

Unfortunately, measuring lower phosphorylation of key signaling molecules following TCR engagement is neither indicative of defective negative or positive selection. Indeed, a body of evidence in the literature documents that relatively similar pathways are leading to both cell fate decisions, but it is the kinetics of activation of those pathways that characterizes the outcome. As such, the kinetics of ERK activation, an essential kinase for thymocyte differentiation have been shown to mainly affect positive selection and thymocyte differentiation. While at in their rebuttal letter, the authors present an ERK phosphorylation blot that was suggested by the reviewer, it is absolutely inconclusive due to poor quality. In addition the presented kinetics would neither correlate with a positive or negative profile as documented previously, thus it would be very hard to argue for or against an ERK signaling defect that would impact positive selection in absence of LKB1. However, if LKB1 is a major regulator of PLCγ1 activity as implied by the authors throughout the manuscript a "massive" defect in ERK-signaling should be observed (PLCγ1 has been shown to impinge on the ERK pathway).

Again, while LKB1 probably modulates PLCγ1 activity, the authors fail to demonstrate that this signaling axis is affecting positive selection. Alternatively, it may however affect cell survival..... In fact it is very much at odds with common practice in the field to stimulate thymocytes with CD3 as well as CD4 or CD8 coreceptors. This would be OK for mature T cell stimulation but not for thymocytes, and certainly not in the purpose to mimic positive selection as this event is triggered by low affinity ligands. A dual stimulation as the authors are performing would be more similar to a high affinity ligand triggering. Interestingly, the possibility that LKB1 could be involved in negative selection, which is more inline with a "death phenotype" and many of the biochemical findings is snidely commented in the authors rebuttal.

While most of the biochemical experiments linking LKB1 to the TCR signaling pathway, was done by gene transfection in cell lines other than thymocytes (Jurkat cells are derived from mature T cells, not from thymocytes and thus do not accurately mimic thymocyte signaling), the more perplexing experiment with freshly harvested thymocytes was conducted in presence of high concentrations (1-25 mM) of Lck inhibitor (figure 4a & suppl Fig 5). It is puzzling because according to the manufacturer (Santa Cruz biotech) the inhibitor is to be diluted in DMSO. The amount of DMSO at 25 mM is probably quite high, yet, the authors do not present any control experiment showing the effects of similar amounts of the vehicle alone. This becomes a major issue as these experiments are providing the fundaments as to the authors claim that Lck, but not Fyn or another Src kinase phosphorylates LKB1. Notably, the authors fail to document that this Lck-induced phosphorylation does indeed lead to augmented LKb1 activity!!!!

Additionally while the authors add confocal imagery to demonstrate LKB1 recruitment to LAT, which is a nice technical prowess that does not add much to the take home message, they fail to adress reviewer 2' s comment about a potential mechanism by which LKb1 regulates PLCγ1-LAT interaction. If not shown experimentally, mechanistical possibilities should alt lest have been discussed.

Thus based on the many more inconclusive results provided, especially as far as examining positive selection is concerned, it is highly suggested that the authors consider alternative explanation to account for the reduced thymocyte numbers in absence of LKB1, such as increased cell death.

Referee 2:

In the revised manuscript, Cao et al. addressed majority of the questions raised by the reviewers. However, concerns still remain and should be addressed:

1. The authors claimed that phosphorylation of Y36, Y261 and Y365 on LKB1 is important for PLCg1 activation, as demonstrated by PLCg1 phosphorylation. A more definitive functional assay for PLCg1 activity is Ca++ flux. Since the authors have already introduced WT and mutant LKB1 into LKB1-/- DP thymocytes, examination of Ca++ flux to support their statement should not be a difficult experiment.

2. To support the statement that LKB1 is directly phosphorylated by Lck at Y36, Y261 and Y365 following TCR stimulation, the authors added new data in Fig. 5D showing that these Y residues are phosphorylated in Jurkat cells following TCR and CD28 engagement. However, the length of stimulation, which was 1 hour, is at odds with a function of LKB1 in TCR proximal signaling, upstream of PLCg1 activation.

The authors should examine phosphorylation at these Y sites at more physiologically relevant time points.

Referee 3:

The authors have sufficiently addressed my comments. The added experiments have substantially added to the impact of the manuscript.

Additional Correspondence by Author 26 February 2011

We thank you and the reviewers for the comprehensive review of our manuscript, and we are encouraged by the facts that two referees are satisfied with our revised manuscript.

There are several points we'd like to clarify regarding to the #1 referee's comments:

1. The role of LKB1 in positive selection is still inconclusive.

As we mentioned in the point-by-point reply, we have analyzed the key events relevant to thymocyte positive selection and we believe that we have generated solid data to demonstrate that LKB1 regulates thymocyte positive selection.

2. The FTOCs experiments are widely inconclusive as the authors did not add exogenous selection ligands.

The phenotype of the AND TCR transgenic mice is sufficient to show that thymocyte positive selection is impaired in the absence of LKB1. Notably, none of the other two referees requires FTOC experiments to verify positive selection, and in fact this referee also thinks the H-Y mouse alone is sufficient to address positive selection.

We aimed to further verify whether the generation of SP population is impaired rather than attempt to check whether LKB1 control positive selection or negative selection, therefore, we did the FTOC

experiments (Figure 1F) without adding exogenous ligands as described in other reports. Interestingly, only marginal amount of CD4 SP were generated in the absence of LKB1, which indicated that the presence of CD4 SP in adult LKB1-deficient mice may be due to accumulation. We will remove Figure 1F if you think this result added little to the manuscript.

The FTOC experiments in Figure 7C and D were done with non-TCR transgenic thymocytes, and of course, no exogenous ligands should be added.

3. To address the role of LKB1 in negative selection.

LKB1 could be involved in the regulation of thymocyte negative selection given that LKB1 regulates TCR signaling. However, this is not a topic of this study.

4. To distinguish between a role for LKB1 in positive selection vs. a role in survival of SP cells.

We agree with the referee that this is a concern although we have distinguished the role of LKB1 in positive selection vs. in survival of pre-selected cells. However, it is practically difficult to address this question because we can't manipulate the cell survival of SP cells if these cells don't exist (in the absence of LKB1). We had included alternative explanation in the revised version.

And more, we notice that some viewpoints and concepts of #1 referee are not matched with the latest progress of the field of thymocyte development. e.g. the relationship between the calcium flux and positive/negative selection; The selection of DP thymocytes could be divided into four steps; the signaling study (phosphorylation analysis) on DP thymocytes with stimulating CD3 instead of CD3 as well as CD4 coreceptor; the role of Bcl-XL on positive selection, and the FTOC experiment to verify positive selection and so on.

We are deeply concerned that this referee may misunderstand our data. We'd like to ask for another referee should you think it is plausible.

We appreciate you and the referees for your nice suggestion and helpful criticism. As detailed in the previous point-by-point reply, the manuscript has been revised to address all the major concerns raised by the referees. We are grateful that the other two referees agree that the manuscript has been improved after the revision. We hope our revised manuscript will be suitable for publication in your distinguished journal.

Thank you very much for your consideration.

Point-by-Point-Response to Referees

Referee 1:

MS# EMBOJ-2010-76341

"LKB1 regulates TCR-mediated PLCγ1 activation and thymocyte positive selection" By Cao., et al

Since the first submission, the authors have undertaken a significant amount of work to improve the quality of the manuscript by attempting to answer the reviewers concerns. Unfortunately, they main findings as to the role of LKB1 in promoting positive selection are still inconclusive or overstated at best and inaccurate at worst. That LKB1 regulates positive selection is one of the main claims of this work as indicated in the title and throughout the manuscript and should therefore be accurately documented.

As we mentioned in the previous point-by-point reply, thymocyte positive selection is accompanied by the upregulation of CD5, CD69 and TCR molecules and induces two key events (the survival and the differentiation). We have shown that 1) LKB1-deficient DP thymocytes were unable to express CD5, CD69 and TCR molecules at a level equivalent to that of their counterparts; 2) LKB1-deficient $CD4^{+}CD8^{low}$ intermediate thymocytes failed to upregulate Bcl-2 and IL-7R molecules (for survival);

3) LKB1-dificient thymocytes failed to upregulate the lineage-specifying factors ThPOK and Runx3 (for differentiation); 4) Both AND TCR Transgenic and non-TCR transgenic mice failed to generate normal SP populations in the absence of LKB1 and 5) LKB1-deficient thymocytes were unable to differentiate further into CD4 or CD8 SP cells even when their survival was prolonged by ectopic expression of the antiapoptotic Bcl-XL protein. Taken together, we think we have solid evidence to support that LKB1 regulates positive selection. Moreover, the attenuated TCR signaling in the absence of LKB1 further supports our conclusion that LKB1 is involved in the thymocyte positive selection.

Thus, the FTOC generated in response to the reviewer's request (Figure 1f) does not prove a defect in selection per se, as the authors did not add exogenous selection ligands (at least nothing the like is mentioned in the figure legends or the methods section). Indeed as the fetal circulatory system is not providing ligands anymore and as endogenous thymic ligands are exhausted after a couple of days, one has to add ligands for the transgenic TCR to the culture in order to observe true positive or negative selection.

The TCR transgenic model system per se is currently believed to be sufficient to verify whether thymocyte positive selection is impaired when an interested gene is ablated (Fu et al, 2009; Rivera et al, 2000). FTOC experiments with exogenous selection ligands are usually used for the analysis of ligand-receptor complex binding affinity and the study of positive selection in an embryonic lethal situation (Daniels et al, 2006; Jameson et al, 1994; Kaneta et al, 2000; Spain et al, 1999), although this kind of experiment is also used to further confirm whether positive or negative selection is affected (Mariathasan et al, 2001).

The fact that AND TCR Transgenic mice failed to generate normal CD4 SP populations in the absence of LKB1 (Figure 1 C, D and E) has already indicated that LKB1 is involved in the regulation of positive selection. We aimed to further verify whether the generation of SP population is impaired rather than attempt to check whether LKB1 control positive selection or negative selection. Therefore, we did the FTOC experiments without adding exogenous ligands as described in other reports, in which no ligand was added (Anderson and Jenkinson, 2007; Jones et al, 2000). Consistent with other studies, a large amount of CD4 SP cells were generated in the wild-type AND TCR Transgenic thymic lobe (which reflects that endogenous thymic ligands worked rather than been exhausted), whereas only marginal amount of CD4 SP was found in the absence of LKB1.

As we mentioned in the previous point-by-point reply, we could not exclude the possibility that LKB1 could also be relevant to negative selection. However, the role of LKB1 on negative selection is not a topic of this study.

(Another accepted method in the field of T-cell ontogeny is to test thymic selection by generating H-Y TCR transgenics on the deficient background of the gene of interest. As the H-Y TCR recognizes a male antigen one can test thymic selection based the presence or absence of T cells in male or female pups). Having less SPs in the LKb1 FTOCs in absence of ligand as the authors observe would be more indicative of increased cell death, especially after 6 days of culture (is the same difference observed at day 0?). This is in fact supported by figure 7c in which a higher proportion of Annexin V stained cells (22.4%) is observed in LKB1-defective FTOCs as opposed to wt cultures (15%). Augmented cell death would actually be more inline with the authors previous observation that increased apoptosis is observed in absence of LKB1. It would thus be possible that Lkb1 is required to promote a post-selection survival signal to differentiated thymocytes as has been described for NFkB.

We agree with the referee on that H-Y TCR transgenic mouse is a good model to study positive selection and negative selection. And in fact, the H-Y TCR transgenic model itself is sufficient to address the positive/negative selection issue without additional FTOC experiments (with adding ligands) (Phee et al, 2010).

It is not surprising that a higher proportion of Annexin V stained cells was observed in LKB1-defective FTOCs because we had shown that LKB1 is required for Bcl-XL expression and thymocyte survival. The FTOC experiments shown in Figure 7 C and D were performed with non-TCR Transgenic mice, and certainly, no exogenous ligands should be added. (This experiment was used to check whether LKB1-deficent DP thymocytes could develop into mature CD4 and CD8 population when their survival is restored by ectopic expression of Bcl-XL).

A similarly concern as for the conclusions of figure 1F is raised for Figure 7D, ectopically expressed Bcl-X cannot possibly increase the proportion of SP because there is no selection ligand

added. Since thymocyte's positive (differentiation) or negative selection (death) is resulting from active TCR engagement, only a post-selection stage would be influenced by transfected Bcl-X, but not the selection process itself in absence of ligand. This is supported by the fact that Bcl-X expression does not change either the proportion of subsets in the wild-type FTOCs. If Bcl-X affected thymic selection one would expect to see a change in the latter.

The FTOC experiments shown in Figure 7 C and D were performed with non-TCR transgenic thymocyte, and again, no exogenous ligands should be added.

Bcl-XL is a well-studied anti-apoptotic protein and had been shown to be required for the survival of DP thymocytes (Chao et al, 1998; Ma et al, 1995). Its role in thymocyte development is not the same as that of Bcl-2, the expression of which is elevated in the positive selecting thymocytes. Bcl-XL was chosen because ectopic expression of Bcl-XL could efficiently rescue DP thymocyte survival in our previous report.

As the FTOCs experiments are widely inconclusive, the authors have so far only shown marginal expression differences of surface receptors CD5, CD69, TCR, CD24 to support their claim of relevance of LKb1 in thymic selection. This is by far to little, especially as the authors have not considered entertaining the sound comment of reviewer 1 as to "include statistical analysis" to better support expression differences of the analyzed receptors. Instead, the authors have introduced FACS measurements of IL-7R and BCL-2, two receptors that are required for survival of differentiated SP cells. Again even though decreased expression of those 2 receptors is measured in absence of LKB1, it is not proof of a bona fide defect in positive selection. Indeed, the authors in their rebuttal (bottom page 1) agree to a multi-phase process of transition from DP to SP ('the survival and the differentiation). What they fail to mention is that there is in fact at least 4 distinct phases (negative and positive selection, lineage commitment as well as survival). The TCR engagement characterizes positive and negative selection, distinct gene expression such as ThPOK or Runx3 accompanies lineage commitment and upregulation of survival markers such as IL-7R denote the last phase.

As we mentioned above, we have analyzed the key events relevant to thymocyte positive selection and we believe that we have generated solid evidence to support that LKB1 regulates positive selection.

There was some misunderstanding here, we said positive selection induces two key events (the survival and the differentiation) in stead of “the transition from DP to SP” induces two key events. In fact, for an individual thymoctye, it undergoes one of three cell fates: positive selection, negative selection or death by neglect, depending on its TCR affinity (Palmer, 2003), rather than “at least 4 distinct phases (negative and positive selection, lineage commitment as well as survival)”.

Unfortunately, measuring lower phosphorylation of key signaling molecules following TCR engagement is neither indicative of defective negative or positive selection. Indeed, a body of evidence in the literature documents that relatively similar pathways are leading to both cell fate decisions, but it is the kinetics of activation of those pathways that characterizes the outcome. As such, the kinetics of ERK activation, an essential kinase for thymocyte differentiation have been shown to mainly affect positive selection and thymocyte differentiation. While at in their rebuttal letter, the authors present an ERK phosphorylation blot that was suggested by the reviewer, it is absolutely inconclusive due to poor quality. In addition the presented kinetics would neither correlate with a positive or negative profile as documented previously, thus it would be very hard to argue for or against an ERK signaling defect that would impact positive selection in absence of LKB1. However, if LKB1 is a major regulator of PLC activity as implied by the authors throughout the manuscript a "massive" defect in ERK-signaling should be observed (PLC has been shown to impinge on the ERK pathway).

We showed that PLCγ1 activity was attenuated but not eliminated in the absence of LKB1, it is understandable that ERK remained partially phosphorylated upon TCR stimulation. In addition, we can not exclude the possibility that LKB1 also suppresses ERK activity through other unknown yet pathways.

Again, while LKB1 probably modulates PLC activity, the authors fail to demonstrate that this signaling axis is affecting positive selection. Alternatively, it may however affect cell survival..... In fact it is very much at odds with common practice in the field to stimulate thymocytes with CD3 as well as CD4 or CD8 coreceptors. This would be OK for mature T cell stimulation but not for thymocytes, and certainly not in the purpose to mimic positive selection as this event is triggered by

low affinity ligands. A dual stimulation as the authors are performing would be more similar to a high affinity ligand triggering. Interestingly, the possibility that LKB1 could be involved in negative selection, which is more inline with a "death phenotype" and many of the biochemical findings is snidely commented in the authors rebuttal.

In fact, the signaling study (phosphorylation analysis) on DP thymocytes was routinely performed by stimulating DP thymocytes with CD3 as well as CD4 coreceptor because of the low surface TCR level on the thymocyte (Delgado et al, 2000; Fu et al, 2009; Naramura et al, 1998). It has been reported that stimulating DP thymocytes with CD3 alone was unable to induce sufficient protein phosphorylation (Luo et al, 2004).

While most of the biochemical experiments linking LKB1 to the TCR signaling pathway, was done by gene transfection in cell lines other than thymocytes (Jurkat cells are derived from mature T cells, not from thymocytes and thus do not accurately mimic thymocyte signaling), the more perplexing experiment with freshly harvested thymocytes was conducted in presence of high concentrations (1-25 mM) of Lck inhibitor (figure 4a & suppl Fig 5). It is puzzling because according to the manufacturer (Santa Cruz biotech) the inhibitor is to be diluted in DMSO. The amount of DMSO at 25 mM is probably quite high, yet, the authors do not present any control experiment showing the effects of similar amounts of the vehicle alone. This becomes a major issue as these experiments are providing the fundaments as to the authors claim that Lck, but not Fyn or another Src kinase phosphorylates LKB1. Notably, the authors fail to document that this Lck-induced phosphorylation does indeed lead to augmented LKb1 activity!!!!

We apologize for our mistake in the labeling of the inhibitor concentrations in Supplementary Figure 5 and the legend to Figure 4 A. The unit should be μM, not mM. We did this experiment as described in previous report (Treanor et al, 2006), DMSO was diluted from 1000 to 25000 folds when we performed these experiments.

Additionally while the authors add confocal imagery to demonstrate LKB1 recruitment to LAT, which is a nice technical prowess that does not add much to the take home message, they fail to adress reviewer 2' s comment about a potential mechanism by which LKb1 regulates PLC-LAT interaction. If not shown experimentally, mechanistical possibilities should at lest have been discussed.

We had discussed the mechanistical possibilities in the revised version (page 15).

Thus based on the many more inconclusive results provided, especially as far as examining positive selection is concerned, it is highly suggested that the authors consider alternative explanation to account for the reduced thymocyte numbers in absence of LKB1, such as increased cell death.

We had also included alternative explanation in the revised version (page 16).

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Referee 2:

In the revised manuscript, Cao et al. addressed majority of the questions raised by the reviewers. However, concerns still remain and should be addressed:

1. The authors claimed that phosphorylation of Y36, Y261 and Y365 on LKB1 is important for PLCg1 activation, as demonstrated by PLCg1 phosphorylation. A more definitive functional assay for PLCg1 activity is Ca++ flux. Since the authors have already introduced WT and mutant LKB1 into LKB1-/- DP thymocytes, examination of Ca++ flux to support their statement should not be a difficult experiment.

We agree with the referee that it would be better to show Calcium flux results, which was also included in our plan initially. However, GFP fluorescence takes the same Emission Max as Fluo-3 AM does, so they cannot be analyzed by FACS instrument simultaneously. In addition, FACS machine with 5 color analysis was currently unavailable to us, leaving us unable to perform such FACS analysis at present. Notably, the phosphorylation site of PLCγ1 we detected is essential for its activation and thus the absence of which should fail to induce intracellular $Ca2^{+}$ flux (Fu et al, 2010; Winslow et al, 2003).

2. To support the statement that LKB1 is directly phosphorylated by Lck at Y36, Y261 and Y365 following TCR stimulation, the authors added new data in Fig. 5D showing that these Y residues are phosphorylated in Jurkat cells following TCR and CD28 engagement. However, the length of stimulation, which was 1 hour, is at odds with a function of LKB1 in TCR proximal signaling, upstream of PLCg1 activation. The authors should examine phosphorylation at these Y sites at more physiologically relevant time points.

The Jurkat cell line we used doesn't express CD4 or CD8, and that is why TCR and CD28 engagement were alternatively used. The more than 1 h-stimulation has been also used in other reports (van den Brink et al, 1999). We will redo this experiment with short stimulation period if the editor agrees (Given that only single round of revision allowed).

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Referee 3:

The authors have sufficiently addressed my comments. The added experiments have substantially added to the impact of the manuscript.

We thank the referee for his/her encouragement and his/her nice suggestions.

2nd Editorial Decision 07 March 2011

Many thanks for sending me the point-by-point response to the referees' re-review of your manuscript. I have now had the chance to discuss the case in depth with an editorial advisor, who is satisfied that your FTOC experiments have been appropriately conducted - in contrary to the negative report of referee 1. I am therefore pleased to be able to tell you that we will be able to accept your manuscript for publication here, pending the remaining minor revisions to address the other concerns of the referees (as you outline in your response): a second round of revision with these kind of relatively minor changes is not a problem.

Can I also ask you to include an Author Contributions statement in your revised manuscript?

I look forward to receiving the revised version of your manuscript.

Yours sincerely,

Editor

The EMBO Journal

2nd Revision - Authors' Response 11 March 2011

As above – in additional correspondence of 26th February.

3rd Editorial Decision 14 March 2011

Many thanks for submitting the revised version of your manuscript. Everything looks fine now, so I am pleased to be able to tell you that we can accept the manuscript for publication in the EMBO Journal - you should receive the formal acceptance message shortly.

Thanks for choosing EMBOJ for publication of this study!

Yours sincerely,

Editor

The EMBO Journal

