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LKB1 inhibition of NF-kB in B cells prevents T folicullar helper cell differentiation and germinal center formation

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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 26 September 2014

I have now heard back from the three referees that assessed your study for EMBO reports. As you will see, although the referees find the study of interest to the field, they also raise various concerns about the conclusiveness of the data, data analysis and presentation. In all, given also that they rate the novelty and general interest of the study as "medium" in the table submitted with their reports, it will be crucial to provide additional data to support your claims for further consideration here.

Given that all referees provide constructive suggestions on how to strengthen the work, I would like to give you the opportunity to revise your manuscript. If the referee concerns can be adequately addressed, we would be happy to accept 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.

From the analysis of the referee reports, the most crucial issues to address would be to causally implicate IL-6 production in GC formation, rule out that spontaneous GC formation is due to autoimmunity or immmunodeficiency, provide further support for the implication of NF-kB in the IL-6 overproduction, analyze the kinetics of LKB1 regulation in WT B cells, and clarify the involvement of apoptosis in KO B cells. In addition, there are a number of controls, issues with data

analysis and presentation that need to be addressed.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

REFEREE REPORTS:

Referee #1:

Interesting work and an interesting model.
Positives are a good definition of the phenotype of the animals with perhaps one important caveat and the somewhat fortuitous result of the B-cells being chimeric for LKB1 deletion so that intrinsic and extrinsic effects can be determined.
Negatives are:
1) relatively significant leap between in vitro cultures of B cells with CD40L/IL4 and in vivo serum IgG. It is not clear to me that the absence of PC in vitro is nothing more than reduced proliferation capacity of the KO cells, such that few progress through sufficient divisions to differentiate into PC. The analysis of the cultures is very confusing. Presenting the % of IgG1+ cells that are YFP+ on day 3 or the % of CD138+ cells that are YFP+ on day 5 (Fig 4) is irrelevant if one doesn't know the % YFP cells in the remainder of the culture at the same time.
3) The T-cell dependent immunization should be measured at time points later than d14 done the normal antibody amounts at that time could be due to extra-follicular plasma cells and not those derived from the GC. Perhaps consider ELISpot assays from BM. Again, as for (2), the proposed theory of why there is a normal NP response only makes sense if there is in fact a normal NP response. The authors should measure the % of NP-reactive B cells within the GC of the immunized mice over time. If they can't do antigen specific staining, they could use Ig-lambda light chain as a surrogate on a C57B6 background.
4) Check mice for autoimmunity. The idea of spontaneous GC does not usually play out; they are almost always self-reactive.
5) The BrdU incorporation studies refer to E6E but should be E6D
6) The inflammatory cytokines are really interesting but present a huge problem to the interpretation. Why IL-6 as the causative, single agent? It is a very mild and modest player in Tfh development, as indicated in the references cited in this report (Eg Eto et al, and Karnowski et al). IL6 is clearly a major inducer of Il21 in in vitro activated T cells, which is again shown here, but in vivo, its role is much less clear. A better experiment would have been to treat the mice with neutralizing anti-Il6 antibody for a period and determine if that rescued the phenotype or better yet, to limit Il6 deficiency to B cells in the KO mice. I appreciate how time consuming that is to do and suggest it only as the ideal.
7) The selection of NFkB as the causative pathway leading to IL6 overproduction is also a curious choice, as I think the authors recognize in their description of the result, where they recognize other possible pathways. Even with these data, shouldn't the experiments have involved a titration of inhibitor rather than an absolute blockade? The inhibitor studies in Fig 7E must contain a wild type control. At the moment there is no point of reference for the degree of inhibition. Currently the results show that NFkB activity contributes to IL-6 production, not that heightened NFkB activity causes increased IL-6.

Overall, a really interesting phenotype but a somewhat disjointed analysis at the end, leaving one uncertain what is causative and what is associative.

Referee #2:

Using conditional KO mice, this study provides evidence that liver kinase B1 (LKB1) is needed for the regulation of GC formation through B cell intrinsic functions that include regulation of Tfh cell-inducing cytokine production. Overall, the study contains a number of new findings regarding LKB1 function that are likely to be of interest to immunologists. However, there are a number of concerns that need to be addressed.

The analysis of CD19-Cre driven deletion of LKB1 in B cells was more confusing than it needs to be, and is spread over 3 figures. It may be better to show cell number, frequency and YFP % for each subset in one figure (or one plus supplement).

The authors see impressive spontaneous GCs (and associated Tfh cell responses) in the BKO mice. However, the GCs are enriched for YFP-ve cells suggesting that the increased activation is not intrinsic to the B cell. Spontaneous GCs could be a consequence of raised cytokine production as they suggest, but could also be secondary to some other form of immunodeficiency. Mixed bone marrow chimera experiments would be a good approach to resolve this issue (the increased IL-6 should still induce more GCs in that setting if the model is correct).

Much of the data from figure 6 (T cell activation) might be included in figure 3 - spontaneous immune response - because the presence of Tfh cells does not provide much additional mechanistic insight.

The authors propose a model where antigen triggers LKB1 downregulation to permit GC induction. It would be useful to show the kinetics of LKB1 regulation in WT B cells (at the least BCR/BCR+CD40 in vitro if not in vivo downregulation).

In fig 5, they characterize B cells from the BKO mice (I presume shaded areas are WT). Do they see differences in these stains in the YFP+ and YFP-ve cells (MHCII CD69 etc)? The BrdU staining (fig 5C and D) is a little messy. 7-AAD+ BrdU-ve cells are shown after a short BrdU pulse, a time point when all dividing cells would be expected to by BrdU+ve. Were doublets tightly excluded because they could cause 7AAD to appear high.

The IL-6 mRNA analysis could all be in one plot with WT, GFP-ve and GFP+ next to each other. Care is needed in interpreting/discussing the contribution of B cell derived IL-6 to the GC response (as opposed to other potential sources, e.g. stromal populations).

The ex vivo plasma cell staining could be in main figure instead of in supplement. In Fig E4D it is not clear that the cells being gated are PCs. Efficient plasma cell recovery may require enzymatic digestion of the spleen tissue. This analysis would be improved by intracellular IgG staining. Fig 4A and 4B might benefit from showing representative FACS plots for both YFP+ and YFP-ve cells. Are the graph figures correct? They are not representative of the FACS plots.
Is LKB1 having an indirect effect on PCs in vivo (given most GC are LKB1+) but direct in vitro (given the PC numbers are not raised in BKO)? What happens to PC numbers after immunization?

To demonstrate that LKB1 is acting via NFkB the authors look at IL-6 production in fibroblasts. They give a lot of emphasis about IL-6 being overproduced by B cells, but there is no direct evidence provided to show that IL-6 production by B cells is the key mechanism driving the spontaneous responses.

Regarding the conclusion that B cell hyperplasia in the KO occurred due to increased apoptosis, Fig E4G is not convincing. How can the authors make this comparison given how few GCB cells are present in control mice? Any total increase in apoptosis is probably a consequence of their being more GCB cells/activated cells rather than being direct consequence of LKB1.

Other minor points
fig E1B, What is 36B4 - a housekeeping gene?
fig E1E, FACS plot not representative of pooled data shown in graph.
fig 2D, what is the blue staining in the IHC? Are the B220 and CD3 stains of sequential sections? Would have been more useful to co-stain as is hard to interpret what you are looking at.
fig 4A, FACS plot impossible to interpret without day 0.
Are IL-4R levels normal on naive BKO cells (given IgG1 and PB response possible defective)?

Referee #3:

T follicular helper (TFH) cells have received a substantial amount of attention recently in both humans and mice. These cells have emerged as a distinct subset/lineage of CD4+ T cells that are

primarily responsible for providing help to B cells during immune responses to T-cell dependent Ag and mediating the differentiation of B cells into memory and plasma cells during GC reactions in secondary lymphoid tissues. Many studies by numerous groups have characterized TFH cells with respect to their surface phenotype, and the roles that cytokines (IL-6, IL-12, IL-21, IL-27), surface molecules (ICOS/ICOS-L, CD40L, SLAM family receptors), signalling molecules (SAP, STAT3), transcription factors (Bcl-6, IRF4, cMAF, STAT3) and different populations of APCs have in their generation, maintenance and/or survival. Although we currently have a substantial understanding of the requirements for generating Tfh cells, this is incomplete and additional studies are required especially to understand the counter-regulation of Tfh formation.

In this current study, Walsh and Teitell have revealed an important T-cell extrinsic role for the kinase LKB1 in controlling Tfh formation. This was achieved by generating mice lacking LKB1 expression in the B-cell lineage and examining these mice for lymphocyte generation and function. Altho LKB1 was not deleted from all B cells, it was apparently deleted from a sufficient proportion of cells to manifest a physiologically-relevant phenotype. Specifically, it was found that while mice lacking B-cell specific LKB1 had increased numbers of GC B cells and IgG1+ switched B cells, this was largely due to effects of both LKB1-sufficient and deficient B cells in terms of proliferation and differentiation. These defects to B-cell differentiation were clearly unrelated to a lack of LKB1 as both LKB1- and LKB1+ B cells exhibited similar features - these effects were attributed to aberrant production of IL-6 by B cells in LKB1-deficient mice - again tho, this was noted for both LKB1- and LKB1+ B cells in these animals. The immediate consequence of this aberrant IL-6 was a massive enhancement in the generation of Tfh cells as revealed by increased % and numbers of these cells as well as elevated expression of key Tfh effector molecules IL-4 and IL-21. As LKB1 regulates NFkB signaling it was concluded that reduced LKB1 function in B cells results in exacerbated IL-6 produciton which acts on CD4 T cell to drive these cells to a Tfh phenotype thereby resulting in increased B cell function (GCs, class switching). Curiously tho, there was little net effect on Ig levels - both total and Ag-specific - mostly due to accelerated apoptosis of the B cells.

There are some interesting findings reported here that confirm the important role of IL-6 in regulating Tfh cells and extend previous work demonstrating that B cells can be a source of IL-6. Several aspects of this work could be improved by addressing a few issues.

1. Several findings report on gene expression levels in population of total B cells or CD4 T cells eg AICDA, IL21, IL4. Likewise, expression of MHC class II, CD86, CD69 was assessed on total CD19+ B cells - It is not really surprising that levels of these transcripts and surface markers are increased in the BKO mice when analysing total cells because GC B cells (which express AICDA, and upregulate CD86 etc) and Tfh cells (expressing IL4, IL21) are increased. It would be more informative to determine whether these genes are over-expressed by BKO GC B cells and BKO Tfh cells vs corresponding cells from normal donors.
2. P8 - there is a comment that "When partitioned into B cell populations by flow cytometry, only GC B cells showed increased apoptosis (10 fold) in BKO-YFP compared to controls spleens (Fig 3E)". Fig 3E shows a very modest increase in caspase+ cells in the GC subset for the BKO vs control and it is no where near 10-fold. Similarly, (p9): "on d3 only 40% of IgG1+ B cells in BKO-YFP cultures were LKB1- YFP+ (Fig 4A)" - this value stated in the figure is 53.7%
3. IL-6 was found to be 300 fold higher in CD43-depleted spleens but only 3-fold increased in the LKB1-deficient compared to LKB1-sufficient B cells in the BKO mice. this suggests that IL-6 might be promoting its own expression ie in an autocrine manner. So expression levels (and secretion values would be very helpful) should be determined for B cells from WT mice and compared to LKB1-deficient and LKB1-sufficient B cells in the BKO mice. it would also be helpful to determine which B cell subset is responsible for this excessive IL-6 production. Follicular or GC B cells would be the logical population as these cells will be the ones interacting with Tfh-type cells.
4. FoxP3 and IL10 were also found to be increased in splenic CD4 T cells from BKO mice vs WT mice, which led to the conclusion that Treg cell differentiation was also increased in the mutant mice. however, it is possible that this reflects T follicular regulatory (Tfr) cells - it would be worth performing FACS analyses for FoxP3 expression amongst the Tfh cells in the BKO vs WT mice.
5. Were elevated levels of IL-6, and some of the other inflammatory factors, also detectable in serum?
6. Corcoran and colleagues recently reported that the B cell transcription factors Oct2 and OBF1 were required for B-cell IL-6 production which then contributes to Tfh formation. Can these factors

be incorporated into the model presented which illustrates how the authors view LKB1 functions to regulate IL-6 production, and subsequently Tfh cells, by B cells?

1st Revision - authors' response 12 January 2015

Response to reviews for revised manuscript EMBOR-2014-39505V1 by Walsh, et al.

Referee #1:

General Comments: Interesting work and an interesting model. Positives are a good definition of the phenotype of the animals with perhaps one important caveat and the somewhat fortuitous result of the B-cells being chimeric for LKB1 deletion so that intrinsic and extrinsic effects can be determined.

General Response: We thank the reviewer for these positive overall comments on our study and chimeric B cell specific LKB1 knockout mouse model.

Comment 1: relatively significant leap between in vitro cultures of B cells with CD40L/IL4 and in vivo serum IgG. It is not clear to me that the absence of PC in vitro is nothing more than reduced proliferation capacity of the KO cells, such that few progress through sufficient divisions to differentiate into PC. The analysis of the cultures is very confusing. Presenting the % of IgG1+ cells that are YFP+ on day 3 or the % of CD138+ cells that are YFP+ on day 5 (Fig 4) is irrelevant if one doesn't know the % YFP cells in the remainder of the culture at the same time.

Response 1: Actually, in original figure E6C (now revised figure E5D), the data show that LKB1-YFP+ B cells proliferate more by day 3 of stimulation than do LKB1+YFP- B cells from the BKO mice, so it is unlikely that LKB1- B cells cannot divide sufficiently to differentiate into PCs. However, in the revised manuscript, we use this data to support the proliferative and activated state of B cells from BKO mice, rather than as evidence against the lack of proliferation for the inability of LKB1- B cells to form PCs in vitro. We removed the in vitro differentiation assay data (original figures 4 and E5C) because of concerns raised by several reviewers for relevance between the B cell purified in vitro assay and in vivo differentiation and have refocused the paper on the role for LKB1 at germinal center (GC) formation. We thank the reviewer for the helpful comment, which is consistent with those made by the other reviewers concerning the in vitro differentiation assay system.

Comment 2: (none provided)

Comment 3: The T-cell dependent immunization should be measured at time points later than d14 done the normal antibody amounts at that time could be due to extra-follicular plasma cells and not those derived from the GC. Perhaps consider ELISpot assays from BM. Again, as for (2), the proposed theory of why there is a normal NP response only makes sense if there is in fact a normal NP response. The authors should measure the % of NP-reactive B cells within the GC of the immunized mice over time. If they can't do antigen specific staining, they could use Ig-lambda light chain as a surrogate on a C57B6 background.

Response 3: We agree that the NP-antibody response at D14 could be due to an increased extra-follicular plasma cell response. As suggested, the NP-IgG1 response at 28 days post-immunization was quantified (revised Figure E4) and no difference between WT and BKO mice was detected, suggesting against an early, purely extra-follicular plasma cell response. The main point of this data is that BKO mice can respond to a T cell dependent antigen, so the GCs are functional.

Comment 4: Check mice for autoimmunity. The idea of spontaneous GC does not usually play out; they are almost always self-reactive.

Response 4: We added an analysis of anti-nuclear antibodies (ANAs) in the serum of mice aged between 6 and 9 months (new Figure E3H), as suggested. There is an increase in the number of

BKO mice that are positive for ANAs compared to littermate control mice (4/19, 21%), but this information alone does not indicate whether increased GC formation is due to an increase in self reactive B cells or that self reactive B cells are increased because they are less efficiently deleted in BKO mice.

Comment 5: The BrdU incorporation studies refer to E6E but should be E6D

Response 5: This has been changed and is now revised Figure E5E, as suggested, and thank the reviewer for catching this error.

Comment 6: The inflammatory cytokines are really interesting but present a huge problem to the interpretation. Why IL-6 as the causative, single agent? It is a very mild and modest player in Tfh development, as indicated in the references cited in this report (Eg Eto et al, and Karnowski et al). IL6 is clearly a major inducer of Il21 in in vitro activated T cells, which is again shown here, but in vivo, its role is much less clear. A better experiment would have been to treat the mice with neutralizing anti-Il6 antibody for a period and determine if that rescued the phenotype or better yet, to limit Il6 deficiency to B cells in the KO mice. I appreciate how time consuming that is to do and suggest it only as the ideal.

Response 6: We focused on aberrant IL-6 expression by LKB1-YFP+ BKO B cells as a contributing factor because of its known role in the activation, differentiation, and expansion of TFH cells that are part of GCs. We did not exclude the possibility that other cytokines or chemokines that are induced in or by LKB1- B cells could also potentially have a contributing role in TFH cell formation and/or maintenance, as suggested by the reviewer. However, none of the additional identified factors have established roles in GC induction or TFH cell differentiation. Additionally, as stated by the reviewer, the role of IL-6 in vivo during TFH differentiation and GC formation is not clear, and it is currently thought that IL-6 and IL-21 in particular may have overlapping or redundant/complementing functions in promoting TFH cell differentiation (Eto et al. 2011. PloS one 6(3):e17739 and Karnowski et al. 2012. JEM 209(110): 2049-2064). Our data does not indicate that IL-6 has a required or unique role in TFH cell differentiation, just that it is sufficient when aberrantly expressed by B cells due to loss of LKB1 expression, which is a unique and important finding given the central role for B cells in starting GC reactions and humoral immunity.

The reviewer's suggestion of administering a neutralizing IL-6 mAb has been used multiple times by others prior to the induction of IL-6 to determine the role IL-6 has during an immune response (Eto et al. 2011. PloS one 6(3):e17739, Prabhakara R et al. 2011. Infection and Immunity 79(12): 5010-5018, Barber DL et al. 2014. J Immunol 192: 676-682). In our model, we would need to administer an IL-6 neutralizing antibody in a dosage that would neutralize pre-existing IL-6 that has already been produced and present in the environment in addition to future IL-6 production, since LKB1 is lost from before birth and the establishment of a functional adaptive immune system. Additionally, administration of mAb would need to continue for a sufficient time period to allow for the involution of established GCs whenever we would intervene. To statistically determine an effect in this setting of IL-6 production from the outset, at 90% power (Cohen's d statistic of 0.5 signal to noise ratio) a sample size of over 60 mice would need to be analyzed in treated and control groups. Even assuming a medium effective size (d of 1), over 20 mice in each group would need to be treated. This would be cost-prohibitive and unlikely effective.

We appreciate that the reviewer isn't suggesting a LKB1/IL-6 B cell double knock-out mouse cross, even though this is the ideal experiment to establish the role for IL-6 as an instigator of the model phenotype. BKO-YFP mice already have 3 independent alleles that have to recombine in a specific configuration to yield analyzable mice (Figure E1A), and adding a fourth allele would generate few mice with the proper genotype to analyze after an extended period of breeding and backcrossing.

Comment 7: The selection of NFkB as the causative pathway leading to IL6 overproduction is also a curious choice, as I think the authors recognize in their description of the result, where they recognize other possible pathways. Even with these data, shouldn't the experiments have involved a titration of inhibitor rather than an absolute blockade? The inhibitor studies in Fig 7E must contain a wild type control. At the moment there is no point of reference for the degree of inhibition. Currently the results show that NFkB activity contributes to IL-6 production, not that heightened NFkB activity causes increased IL-6.

Response 7: There are many pathways that regulate IL-6 expression and we explored the key reported pathways in WT and LKB1- MEFs. Our results show that the only pathway that is activated or induced in LKB1- MEFs is the NF-kB pathway (revised Figures 5 and E7). Therefore, we assessed NF-kB activity in LKB1- B cells and determined that it was also increased in LKB1-YFP+ B cells from BKO mice (revised Figure 5F). Our results are consistent with a role for NF-κB in the regulation of IL-6 (Lintermann TA and Baltimore D. 1990. Mol Cell Biol 10: 2327-2334). It has also been demonstrated in multiple mouse models that loss of negative NF-kB regulation results in increased production of IL-6 in B cells (Chu Y et al. 2011. Blood 117(7): 2227-2236; Lin AE et al. 2013. Nat Immuno 14(1): 27-33) and other immune cells, such as in miR146-/- mice (Boldin MP et al. 2010. JEM 208(6): 1189-1201; Zhao JL et al. PNAS 108(22): 9184-9189; and Zhao JL et al. 2013. eLIFE 2:e00537). Additionally, a recent December, 2014 study detailed regulation of NF-kB by LKB1 in macrophages, with a focus on IL-6 production (Liu Z et al. 2014. J Biol Chem: jbc.M114.616441.)

Finally, our inhibitor studies require a WT control to give a frame of reference for reduced IL-6 expression upon NF-kB inhibition and we have placed this control into revised Figure 5E, as well as a titration of the NF-kB inhibitor on KO MEFs in revised Figure E7F, as suggested. Indeed, IL-6 gene expression is repressed in a dose-dependent manner by an NF-kB inhibitor, suggesting that the level of IL-6 expression correlates with the level of NF-kB activity, which is consistent with the model.

Referee #2:

General Comments: Using conditional KO mice, this study provides evidence that liver kinase B1 (LKB1) is needed for the regulation of GC formation through B cell intrinsic functions that include regulation of Tfh cell-inducing cytokine production. Overall, the study contains a number of new findings regarding LKB1 function that are likely to be of interest to immunologists.

General Response: We thank the reviewer for these positive overall comments on our study.

Comment 1: The analysis of CD19-Cre driven deletion of LKB1 in B cells was more confusing than it needs to be, and is spread over 3 figures. It may be better to show cell number, frequency and YFP % for each subset in one figure (or one plus supplement).

Response 1: The initial characterization of Cre-driven LKB1 deletion in B cells and its effect on homeostasis for B and T cells has been condensed into revised Figure 1 and Figures E1 and E2, as suggested.

Comment 2: The authors see impressive spontaneous GCs (and associated Tfh cell responses) in the BKO mice. However, the GCs are enriched for YFP-ve cells suggesting that the increased activation is not intrinsic to the B cell. Spontaneous GCs could be a consequence of raised cytokine production as they suggest, but could also be secondary to some other form of immunodeficiency. Mixed bone marrow chimera experiments would be a good approach to resolve this issue (the increased IL-6 should still induce more GCs in that setting if the model is correct).

Response 2: Indeed, the data show that GC formation is not restricted to LKB1-YFP+ B cells. Increased MHC class II expression (revised Figure 3B) and IL-6 expression and secretion (revised Figure 4A, 4B), both markers of cell activation, are intrinsic to LKB1- B cells. By contrast, increased expression of CD69 (revised Figure E5B) and CD86 (revised Figure 3B), also markers of cell activation, occur in both LKB1+ and LKB1- B cells, consistent with cell extrinsic activation, likely related to the raised cytokine environment in BKO mice.

The BKO model is based on B lineage specific but incomplete CD19-Cre deletion (Schmidt-Supprian M and Rajewsky K. 2007 Nat Immunol 8: 665-668). It behaves like a mixed BM chimera already because some CD19+ B cells express Cre-recombinase (YFP+) and some do not express Cre-recombinase (YFP-), effectively generating a competition between LKB1+ and LKB1- B cells in the same mice. Quantitatively, ~20% of CD19+ B cells retain LKB1 expression in BKO-YFP, based on expression of YFP in CD19+ B cells in WT-YFP controls (Figure 1A). By generating a mixed BM chimera with BM from BKO mice and additional WT BM cells, we would effectively reduce the pool of LKB1-YFP+ mature B cells in the chimeric animals but still establish the exact same competition model the BKO mice already exhibit. This would not help to determine whether GC formation is secondary to immunodeficiency.

Comment 3: Much of the data from figure 6 (T cell activation) might be included in figure 3 - spontaneous immune response - because the presence of Tfh cells does not provide much additional mechanistic insight.

Response 3: In the revised manuscript, the analyses of all expanded GC populations are included in revised Figure 2, as suggested. Additionally, the analysis of activated lymphocytes is provided in revised Figure 3 instead of two separate figures split into B cell and T cell subpopulations, as suggested.

Comment 4: The authors propose a model where antigen triggers LKB1 downregulation to permit GC induction. It would be useful to show the kinetics of LKB1 regulation in WT B cells (at the least BCR/BCR+CD40 in vitro if not in vivo downregulation).

Response 4: Our model is that signaling from the B cell receptor phosphorylates LKB1 at specific residues, such as S431, which has been shown to be inhibitory for LKB1 function (Zheng B et al. 2009. Mol Cell 33(2): 237-247; Esteve-Puig R et al. 2009. PLos One 4:e4771; and Lo AK-F et al. 2013. J Path 230: 336-346). Data consistent with our model has been added in revised Figure 6 and shows that when B cells are stimulated with an F(ab2) fragment against surface IgM there is an increase in the phosphorylation of ERK1/2 that temporally coincides with an increase in phosphorylation of LKB1 at S431, which would transiently result in LKB1 inactivation with antigen stimulation. Further studies in this more physiological setting, rather than LKB1 knockout, are ongoing and beyond the current scope of study that is focused on the BKO model.

Comment 5: In fig 5, they characterize B cells from the BKO mice (I presume shaded areas are WT). Do they see differences in these stains in the YFP+ and YFP-ve cells (MHCII CD69 etc)? The BrdU staining (fig 5C and D) is a little messy. 7-AAD+ BrdU-ve cells are shown after a short BrdU pulse, a time point when all dividing cells would be expected to by BrdU+ve. Were doublets tightly excluded because they could cause 7AAD to appear high.

Response 5: Expression of MHC class II (revised Figure 3B) is elevated in LKB1-YFP+ B cells compared with LKB1+YFP- B cells from BKO mice, while the expression of CD86 (revised Figure 3B) and CD69 (revised Figure E5B) are similar between LKB1- and LKB1+ B cells but elevated compared to WT-YFP control B cells (revised Figure 3A). This analysis has been added to revised Figure 3.

BrdU staining has been re-assessed and gating has been tightened to ensure doublets are excluded, as suggested. B cells are freshly isolated from un-stimulated mice when pulsed with BrdU; therefore, BrdU positivity reflects the proportion of B cells that were proliferating in vivo at the time of harvest. There could be B cells that are strongly 7-AAD+ (indicating G2/M phase of the cell cycle) that are not BrdU positive because they have already passed S phase during the short 30 min BrdU pulse time. Data more clearly indicating B cell BrdU incorporation (proliferation) for WT-YFP and BKO-YFP B cells, as well as for LKB1-YFP+ and LKB1+YFP- B cells from BKO mouse spleens are shown in revised Figure 3D.

Comment 6: The IL-6 mRNA analysis could all be in one plot with WT, GFP-ve and GFP+ next to each other. Care is needed in interpreting/discussing the contribution of B cell derived IL-6 to the GC response (as opposed to other potential sources, e.g. stromal populations).

Response 6: We thank the reviewer for this suggestion, which is similar to one made by reviewer #3 comment 3 (below), and have assessed IL-6 transcript expression for WT-YFP B cells and LKB1+YFP- B cells and LKB1-YFP+ B cells from BKO mice in the same experiment in revised Figure 4A. Also, we have added IL-6 secretion data for these B cell subpopulations to clearly show that increased IL-6 is originating almost exclusively from LKB1-YFP+ BKO B cells in revised Figure 4B, as suggested.

Comment 7: The ex vivo plasma cell staining could be in main figure instead of in supplement. In Fig E4D it is not clear that the cells being gated are PCs. Efficient plasma cell recovery may require enzymatic digestion of the spleen tissue. This analysis would be improved by intracellular IgG staining. Fig 4A and 4B might benefit from showing representative FACS plots for both YFP+ and YFP-ve cells. Are the graph figures correct? They are not representative of the FACS plots.

Is LKB1 having an indirect effect on PCs in vivo (given most GC are LKB1+) but direct in vitro (given the PC numbers are not raised in BKO)? What happens to PC numbers after immunization?

Response 7: Efficient recovery of and flow cytometry gating on isolated plasmablasts and plasma cells from BM and spleen was done based on our experience in this area and on many papers that have detailed (1) isolation and (2) recovery procedures and (3) B220 plus CD138 staining and gating parameters for the identification of PBs and PCs (Cantor, J et al. 2009. Nat Immunol 10: 412-419; Erazo A et al. 2007. Immunity 26(2): 191-203; DiLillo DJ et al. 2008. J Immunol 180: 361-371; and Quemeneur L et al. 2008. Blood 111(5); 2714-2724).

Figure 4 from the original manuscript has been removed because of comments from all reviewers regarding the interpretation of the in vitro differentiation assay compared to the in vivo process of PC differentiation. The inciting event for the formation of the expanded GCs in BKO mice is LKB1 deletion in a subset of B cells and this is the main focus of the revised manuscript.

Comment 8: To demonstrate that LKB1 is acting via NFkB the authors look at IL-6 production in fibroblasts. They give a lot of emphasis about IL-6 being overproduced by B cells, but there is no direct evidence provided to show that IL-6 production by B cells is the key mechanism driving the spontaneous responses.

Response 8: In revised Figure 4C, the role of aberrant IL-6 production by BKO B cells is established using an in vitro co-culture assay in which naïve BKO B cells cultured with naïve WT CD4+T cells induced the expression of IL-21, a key cytokine for TFH cell differentiation and a canonical cytokine of TFH cells (Crotty S. 2011. Annu Rev Immunol 12(15): 621-663). By contrast, neither CD40L/IL-4 activated WT B cells nor naïve WT B cells induced IL-21 expression in the co-culture system. Furthermore, addition of IL-6R mAb to these co-culture assays reduced IL-21 induction by BKO B cells by ~60%, supporting a role for IL-6 production from BKO B cells in inducing the IL-21 response in naïve CD4+ T cells that has been shown to activate, differentiate, and expand TFH cells.

Comment 9: Regarding the conclusion that B cell hyperplasia in the KO occurred due to increased apoptosis, Fig E4G is not convincing. How can the authors make this comparison given how few GCB cells are present in control mice? Any total increase in apoptosis is probably a consequence of their being more GCB cells/activated cells rather than being direct consequence of LKB1.

Response 9: We concur with the reviewer that an increase in apoptosis in BKO spleens is a logical outcome from an increased number of GC B cells. This is shown in revised Figures 2E and 2F in which only GC B cells display an increased number of cleaved caspase 3 positive cells amongst all of the mature B cell subsets compared to WT controls. Our point for this data is to show that one explanation for why BKO mice display increased GCs but not increased PBs/PCs (revised Figure E3D) is because there is heightened apoptosis of GC B cells, and this is at least one mechanism for how BKO mice handle this B cell hyperplasia. We do not have data showing that this apoptosis is a direct consequence of LKB1 loss, as both LKB1+YFP- and LKB1-YFP+ B cells stain positive for cleaved caspase 3 (revised Figure E3G), nor do we state that increased apoptosis is a direct result of loss of LKB1 in B cells in the paper. The two most logical reasons for this apoptosis is (1) lack of a required energetic transition to glycolysis from OXPHOS when B cells are activated by LKB1 loss (Doughty CA et al. 2006. Blood 107(11): 4458-4465) and (2) lack of a selecting antigen in these un-stimulated mice to rescue GC B cells from FAS-FASL induced apoptosis (Rothstein TL et al. 1995. Nature 374:163-165). We cited both of these well-established mechanisms for GC B cell apoptosis in the text.

Other minor points:

Comment 10: fig E1B, What is 36B4 - a housekeeping gene?

Response 10: *36b4*, also called *Rplp0*, is a housekeeping gene that is used by many groups as a qRT-PCR control (Akamine R et al. 2007. J Biochem Biophys Methods 70: 481-486).

Comment 11: fig E1E, FACS plot not representative of pooled data shown in graph.

Response 11: In Figure E1E, the flow plot has been changed to one that is more representative of the pooled data. The results show that BKO spleens have a lower frequency of B-1a B cells in the setting of a 2-fold increase in total splenocytes, which results in a similar number of B-1a B cells in WT and BKO spleens.

Comment 12: fig 2D, what is the blue staining in the IHC? Are the B220 and CD3 stains of sequential sections? Would have been more useful to co-stain as is hard to interpret what you are looking at.

Response 12: The blue stain is a hematoxylin counterstain that is standard for IHC stained slide sections and used to show the position of cell nuclei, similar to DAPI use in immunofluoresence microscopy. The stains are sequential sections from the same tissue. Co-staining was not done because the counterstains typically are brown and red in color, which are very hard to distinguish visually in the same stained tissue section. The main point of these serial stained sections was to show how the tissue architecture is disrupted by a robust T cell expansion and increased numbers of very large GCs in BKO mouse spleens.

Comment 13: fig 4A, FACS plot impossible to interpret without day 0.

Response 13: All three expert reviewers have commented in one manner or another that interpreting results from the in vitro differentiation assay shown in original Figure 4 to the in vivo differentiation of LKB1- B cells is difficult for multiple reasons. Therefore, original Figure 4 has been removed from the revised manuscript, and the revised submission has been more specifically focused on the role for LKB1 at the start of the GC reaction than at its termination.

Comment 14: Are IL-4R levels normal on naive BKO cells (given IgG1 and PB response possible defective)?

Response 14: Yes, expression of IL-4R was comparable between naïve WT-YFP and BKO-YFP B cells by flow cytometry (MFI of CD19+ B cells: WT-YFP 339±18.8 and BKO-YFP 378±44.9 n=4 each).

Referee #3:

General Comments: In this current study, Walsh and Teitell have revealed an important T-cell extrinsic role for the kinase LKB1 in controlling Tfh formation……. There are some interesting findings reported here that confirm the important role of IL-6 in regulating Tfh cells and extend previous work demonstrating that B cells can be a source of IL-6. Several aspects of this work could be improved by addressing a few issues.

General Response: We thank the reviewer for these positive overall comments on our study.

Comment 1: Several findings report on gene expression levels in population of total B cells or CD4 T cells eg AICDA, IL21, IL4. Likewise, expression of MHC class II, CD86, CD69 was assessed on total CD19+ B cells - It is not really surprising that levels of these transcripts and surface markers are increased in the BKO mice when analysing total cells because GC B cells (which express AICDA, and upregulate CD86 etc) and Tfh cells (expressing IL4, IL21) are increased. It would be more informative to determine whether these genes are over-expressed by BKO GC B cells and BKO Tfh cells vs corresponding cells from normal donors.

Response 1: qRT-PCR was not used in total B cell or CD4+ T cell populations to determine aberrant expression levels of subpopulation specific transcripts, which would not be revealed since the subpopulations are so different between WT and BKO mice, as indicated by the expert reviewer. Where biomarker/gene expression profiling did reveal a difference due to loss of LKB1 was for specific activation markers, as suggested by the reviewer. For this, MHC class II protein (revised Figure 3B) and IL-6 transcripts (revised Figure 4A) were elevated in LKB1-YFP+ B cells compared to LKB1+YFP- B cells from BKO mice, which is the optimal control since both B cell subsets come from the same mouse and therefore reflects the effect of LKB1 loss on the expression of these proteins/genes. By contrast, activation markers CD69 (revised Figure E5B) and CD86 (revised Figure 3B) were similarly elevated between LKB1- and LKB1+ B cell subpopulations compared to

WT-YFP mouse B cells, which is consistent with cell extrinsic activation, likely related to the raised cytokine environment in BKO mice.

Comment 2: P8 - there is a comment that "When partitioned into B cell populations by flow cytometry, only GC B cells showed increased apoptosis (10 fold) in BKO-YFP compared to controls spleens (Fig 3E)". Fig 3E shows a very modest increase in caspase+ cells in the GC subset for the BKO vs control and it is no where near 10-fold. Similarly, (p9): "on d3 only 40% of IgG1+ B cells in BKO-YFP cultures were LKB1- YFP+ (Fig 4A)" - this value stated in the figure is 53.7%

Response 2: Figure 3E in the original manuscript included two graphical representations of cleaved caspase 3+ B cells. The left graph showed the frequency (in percentage) of cleaved caspase 3+ B cells in each subpopulation analyzed from WT and BKO mouse spleens, whereas the graph on the right showed the actual number of cleaved caspase 3+ B cells in each subpopulation. We agree that showing both representations was confusing because the left graph showing frequency did not take into account the number of cells in each subpopulation, which was very different, for example, for GC B cells in WT versus BKO mice. The text only referred to the number of B cells in each subpopulation that showed increased cleaved caspase 3, which was $0.86x10^5 \pm 0.38x10^5$ and $8.7x10^5 \pm 4.3x10^5$ for GC B cells from WT-YFP and BKO-YFP spleens, respectively. Therefore, we removed the graph of the frequency of cleaved caspase 3+ B cells and re-drew the graph quantifying the number of cleaved caspase 3+ B cells in each subset of WT and BKO mice in revised Figure 2E to reduce this confusion.

Figure 4 from the original manuscript has been removed in the revised version because of concerns raised by all reviewers regarding the use of in vitro differentiation assay results to interpret in vivo results.

Comment 3: IL-6 was found to be 300 fold higher in CD43-depleted spleens but only 3-fold increased in the LKB1-deficient compared to LKB1-sufficient B cells in the BKO mice. this suggests that IL-6 might be promoting its own expression ie in an autocrine manner. So expression levels (and secretion values would be very helpful) should be determined for B cells from WT mice and compared to LKB1-deficient and LKB1-sufficient B cells in the BKO mice. it would also be helpful to determine which B cell subset is responsible for this excessive IL-6 production. Follicular or GC B cells would be the logical population as these cells will be the ones interacting with Tfh-type cells.

Response 3: We thank the reviewer for this suggestion and IL-6 gene expression and protein secretion has now been compared in the same experiment between WT B cells and LKB1+YFP- B cells and LKB1-YFP+ B cells from BKO mice. Revised Figure 4A shows Il6 mRNA expression in these three populations and revised Figure 4B shows IL-6 protein secretion from these populations by ELISA. This data also addresses a similar point raised by reviewer #2 comment 6, above.

Data in revised Figure 4A and 4B show that IL-6 transcription and secretion is intrinsic to B cells that lack LKB1 expression. Our results in LKB1- MEFs (revised Figure 5) show that increased IL-6 production is intrinsic to the loss of LKB1 in non-B cells as well. Our data show that all mature B cell subpopulations (e.g. TR, FO, MZ, and GC) contain both LKB1-YFP+ and LKB1+YFP- components, necessitating isolation of each subpopulation by extensive sorting from numerous, not necessarily littermate mice to try to determine which subset produces IL-6. The MEF result suggesting LKB1 loss could potentially activate IL-6 expression in any B cell subset, coupled with the high expense and pooled, likely impure samples required for generating LKB1-YFP+ and LKB1+YFP- B cell subsets, blocked us from addressing this potentially interesting question, as suggested.

Comment 4: FoxP3 and IL10 were also found to be increased in splenic CD4 T cells from BKO mice vs WT mice, which led to the conclusion that Treg cell differentiation was also increased in the mutant mice. however, it is possible that this reflects T follicular regulatory (Tfr) cells - it would be worth performing FACS analyses for FoxP3 expression amongst the Tfh cells in the BKO vs WT mice.

Response 4: We thank the reviewer for this excellent suggestion. Additional analysis of FoxP3+ T cells by flow cytometry was performed as suggested and is presented in revised Figure E6D. The data show a statistically significant increase in the number of CD4+FoxP3+ Treg cells in the spleens of BKO-YFP mice compared to WT-YFP control spleens. By contrast, there is not a corresponding

increase in the CD4+PD-1+CXCR5+FoxP3+ Tfr cell population. The absence of an expanded Tfr population is not too surprising considering the suppressive effect Tfr cells are thought to have on GC development (Linterman MA et al 2011. Nat Med 17: 975-982; Chung Y et al. 2011. Nat Med. 17: 983-988) and the BKO mice have robust GCs. Furthermore, the origin of Tfr cells has been attributed to the thymus as the source for these natural regulatory cells and not as an induced population from naïve, peripheral CD4+ T cells (Wollenberg I et al. 2011. JI 187: 4553-4560; Linterman MA et al. 2011. Nat Med 17: 976-982), which is consistent with the results from BKO mice.

Comment 5: Were elevated levels of IL-6, and some of the other inflammatory factors, also detectable in serum?

Response 5: Levels of IL-6 in serum from both naïve WT and naïve BKO mice were below the limits of IL-6 detection by ELISA.

Comment 6: Corcoran and colleagues recently reported that the B cell transcription factors Oct2 and OBF1 were required for B-cell IL-6 production which then contributes to Tfh formation. Can these factors be incorporated into the model presented which illustrates how the authors view LKB1 functions to regulate IL-6 production, and subsequently Tfh cells, by B cells?

Response 6: Yes, the recently reported regulation of IL-6 production in B cells by Oct2 and OBF-1 by Corcoran and colleagues (Karnowski et al. 2012. JEM 209(11): 2049-2064) is consistent and can be combined with our data to give a more complete model for how IL-6 gene expression is regulated in B cells under different types of stimulated responses. This information is expanded in the Discussion section of the revised manuscript, as suggested. PAMPs, such as influenza infection in vivo, or LPS or CpG stimulation in vitro, could induce a robust TLR-dependent induction of IL-6 through Oct2 and OBF-1 regulated expression as shown in Karnowski et al. Signaling mainly through the BCR and not TLRs by antigen could also have a similar, distinct, or partially overlapping pattern and induced transcriptional response mechanism for activating IL-6 production to facilitate TFH cell differentiation and subsequent GC formation. One mechanism could involve the inactivation, by phosphorylation, of LKB1 downstream of BCR signaling and subsequent activation of NF-kB and Il6 transcriptional induction. Consistent with a more comprehensive model, WT CD43- B cells stimulated for 24 hours with 10 μg/ml LPS secretes ~17 fold more IL-6 than LKB1-YPF+ B cells from BKO mice over 24 hours (814 ± 22.5 pg/ml vs. 46 ± 12.8 pg/ml, respectively). Having multiple mechanisms to regulate IL-6 production provides B cells a way to elicit graded responses of cytokine production depending on the specific antigenic stimuli encountered.

2nd Editorial Decision 04 February 2015

Thank you for your patience while your revised manuscript was seen by the original three referees. As you will see below, although referee 3 is now happy, referees 2 and especially 1 have several outstanding issues. Both feel that your data do not fully justify the claims made.

Given the overall interest of the findings, I would like to open an exceptional second round of revision, which we do not usually allow. Several of the issues you will see raised in the reports of referees 1 and 2 can be addressed by more careful wording. However, I do feel some issues need to be experimentally addressed in order not to water down the message of the study excessively.

- It would be important to better characterize the IL-21 producing cells you denominate Tfh, with which both referees take issue. Referee 2 mentions high Bcl6 expression and a specific surface marker, such as CXCR5, as better surrogates for determining whether they are indeed Tfh cells.

- Points of reference need to be provided in several places of the manuscript (IL-21 production as compared to bona fide Tfh cells; cytokines compared to LPS-stimulated macrophages) in order to allow the reader to interpret the significance of the findings.

- If feasible, improve the analysis of apoptosis in BKO and control mice, as requested by referee 2. Otherwise this section would need toning down, which would detract from the overall impact of the study and would be a pity.

- Provide quantification of 3 or more independent experiments for figure 6.

- Show the requested staining for figure E4.

In addition, please go through the study one more time and ensure the claims made are supported by the data provided. Experimentally addressing some of the issues raised by referee 1 would be out of the scope of the present study, such as the elucidation of the cell extrinsic role of LKB1 or providing support for its role as a central regulator of T cell-dependent humoral immunity, but please rephrase wherever necessary. Note also that we would not insist on the in vivo IL-6 blockade experiment.

I look forward to seeing a revised form of your manuscript when it is ready.

REFEREE REPORTS:

Referee #1:

Facts:
• Hyper-responsive B cells when deficient for LKB1
• Germinal centers in spleens of mice not deliberately immunized
• BKO B cells (or more precisely, CD43- spleen cells from mice with splenamegaly) show elevated cytokine production of multiple cytokines, chemokines.
• Serum titres before and after TI and TD immunization are normal.
• LKB- B cells do not compete effectively with WT in GC
• Tfh are expanded in vivo.
• LKB1- B cells can promote T cell activation in vitro.
• LKB1- B cells co-incubated with anti-CD3 activated T cells induce an amount of IL21 and this is reduced by blocking IL6.
• LKB1 MEFs show hyper-activity of NFkB as do LKB1 B cells
• IL6 production from LKB1- MEKs can be reduced by inhibiting NFkB but remains above WT amount.

Issues remaining:
Main conclusions remain unproven. Specifically:
Title conclusion is not proven - showing reduction in IL6 in tissue culture MEFs when NFkB is restored is not the same as showing Tfh and GC formation in vivo.
IL6 is not shown to promote Tfh cell differentiation and expansion to support a 100-fold increase in steady state GC in vivo
Blockade of secreted IL6 from BKO B cells does inhibit IL21 expression but is not shown to disfavour Tfh differentiation and expansion.
It remains unclear to me what the demonstrated cell extrinsic role for LKB1 in B cells is that controls Tfh cell differentiation and GC formation.
It has also not been shown that LKB1 is a central regulator of T cell dependent humoral immunity since the one TD response studied is apparently completely normal.

This is a very dense manuscript to read and the nomenclature chosen is cumbersome but I can't think of an alternative. I think the facts as listed are well supported by the data. I disagree with the conclusions that are drawn as, to me, they are significant over-reach as indicated above. The model (Fig 7) is a bit simplistic as it fails to explain why LKB1- B cells are not equally represented in the GC.
I would also suggest the argument against IL6 blocking in vivo is a bit spurious. The authors could, for example, clear all existing GC by anti-CD40L treatment and then begin treatment in one group with anti-IL6 and another with an isotype control. If they have done any kind of a time course of GC onset in the mice (have they?) then they will now how long the maximum is they have to wait. My

guess would be 2-3 weeks. I doubt they need 90. I doubt they need 20. 10 would probably do as it is a +/- assay. But if they don't want to do it, fine by me.

Other minor issues:
Scales are often presented without any point of reference. SO for example, the induction of IL21 in the T cell cultures. Is that a lot, a little, equal to a Tfh? The amount of cytokine detected in E6A - same questions relative to LPS macrophage for example. As they are, it is very difficult to know the significance of these numbers. Clearly more than WT, but enough for the proposed purposes/outcomes?
I would encourage the authors to show the staining for GC and NP+GC+ cells in E4. This is important but there is no data presented other than the summary.
Discussion cold be shorter, given issues outlined above.

Referee #2:

In their revised manuscript the authors have addressed many of the earlier concerns. A few remaining points:
1. The authors argue based on the data in figure 2 that BKO GC B cells are undergoing a remarkable 10-fold more apoptosis than control (GC) B cells. Despite this, the BKO mice have at least 10-fold more GC B cells. Without seeing the purity of the GC B cells isolated from the two types of mice I remain unconvinced that the BKO cells have higher apoptosis. Rather, given the much greater frequency of GC B cells in the DKO mice and thus the much improved ability to purity them (they are almost non-existent in the control mice making it very unclear how they are really purified), taken together with the well established fact that GC B cells die extremely rapidly in vitro, it seems much more likely that the difference observed reflects a difference in the amount of 'real' GC B cells vs other (follicular etc) B cells in the 'purified' samples that are being compared. Other approaches would be needed if the authors really want to make this point such as comparing apoptotic cell frequencies in BKO and control mice with equivalently sized GCs by in situ TUNEL staining (or by FACS if mice with equivalent total GC responses can be generated).
2. It is stated in the abstract and in the results and figure 4 legend that BKO B cells induce more 'Tfh cell differentiation' based on an in vitro assay. Production of Tfh cells in vitro remains a 'holy grail' of the Tfh field and thus is not a remark to be taken 'lightly'. For a T cell to be termed a Tfh it minimally needs to be shown to have high expression of Bcl6 and at least some unique marker of this cell subset such as CXCR5. IL21 expression is not considered sufficiently Tfh restricted. The authors can state that BKO B cells can induce IL21-secreting T cell differentiation but they should not state they they can induce Tfh cell differentiation based on the in vitro data.
3. The gene name of 36b4 and that it is a housekeeping gene should be stated in the methods.
4. In figure 6 it should be indicated how many blots are represented by the data shown. Is the second peak at 15 min reproducible?

Referee #3:

The authors have adequately addressed all of the issues raised following the original critique. As such, the manuscript is greatly improved.

2nd Revision - authors' response 06 March 2015

Re: Response to reviews for revised manuscript EMBOR-2014-39505V2 by Walsh, et al.

Thank you for the opportunity to respond to the remaining comments from expert reviewers and for your insightful editorial guidance. We are grateful that the overall interest of the study was recognized and we are allowed a second revision. We responded to each of the re-review comments and provide a revised manuscript that addresses each specific comment.

As you indicated, we have more carefully worded specific areas of the text to make certain that claims made are strongly supported by the data. We appreciate your recognition that some of the issues raised by referee 1 are beyond the scope of the current study. We have addressed all of the specific issues you pointed to in your decision letter as follows:

Editor Comment 1: It would be important to better characterize the IL-21 producing cells you denominate Tfh, with which both referees take issue. Referee 2 mentions high Bcl6 expression and a specific surface marker, such as CXCR5, as better surrogates for determining whether they are indeed Tfh cells.

Response 1: In Figure 2C, there is an increase in the CD4+PD-1high CXCR5high TFH cell population in BKO-YFP mice with a concordant increase in expression of TFH canonical cytokines *Il21* and *Il4*. In vitro co-culture studies show that IL-6 secreted by Lkb1- B cells from BKO mice induced the expression of *Il21* from CD4+CD62L+ T cells. These two cytokines, in combination, have been shown to be sufficient induce TFH cell differentiation in vivo (Karnowski A et al. 2012 J Exp Med; Eto D et al. 2011 PLoS One). Our in vitro co-culture assay identified *Il21* induction by 48 hours, which provides evidence that two TFH cell differentiating cytokines can be induced by BKO B cells, our main point for this assay. We appreciate that we have not broken open the field for in vitro production of TFH cells in vitro, and our text was not intended to indicate this but was nevertheless misleading in this regard. Therefore, we have changed the text to describe the results of this assay as BKO B cells inducing *Il21* expression in naïve CD4+ T cells in an IL-6 dependent manner and removed text indicating TFH cell differentiation in vitro based on this assay alone, which is an appropriate modification. We thank the editor and both referees for pointing out how our text could be misconstrued in this way.

Editor Comment 2: Points of reference need to be provided in several places of the manuscript (IL-21 production as compared to bona fide Tfh cells; cytokines compared to LPS-stimulated macrophages) in order to allow the reader to interpret the significance of the findings.

Response 2: Additional points of reference are now provided, as suggested. We compared the amount of *Il21* transcript produced during the in vitro co-culture experiment with the amount of *Il21* produced by both the CD4+ pool of T cells from BKO mice as well as from purified LCMV-induced TFH cells. The inclusion of CD4+ T cells from BKO spleens is appropriate as it is as close to an in vivo comparison as possible. This data is provided in revised Figure 4D. Also, for a logical subset of inflammatory cytokines that are increased in BKO B cells compared to WT B cells, we compared cytokine secretion to WT B cells stimulated with the inflammatory stimulus LPS as well as to bone marrow derived macrophages stimulated with LPS. This data was added to revised Figure S2B.

Editor Comment 3: If feasible, improve the analysis of apoptosis in BKO and control mice, as requested by referee 2. Otherwise these section would need toning down, which would detract from the overall impact of the study and would be a pity.

Response 3: We compared the frequency of apoptotic GC B cells in naïve BKO mice to that of T cell-dependent NP-CGG antigen stimulated GC B cells at day 10 in littermate control (LMC) WT mice. The data show that the number of caspase3+ B220+GL7+ GC B cells in naïve BKO mice is ~6-fold higher than in LMC mice on day 10 post-immunization, indicating that the GC B cells are undergoing apoptosis at a higher rate than GC B cells induced by specific antigen. This data has been added to revised Figure 2F.

Editor Comment 4: Provide quantification of 3 or more independent experiments for figure 6.

Response 4: The western blot analysis for LKB1 activity has been repeated, quantified, and added to revised Figure 6. An additional control, treatment with the MEK inhibitor U0126, was added to emphasize the phosphorylation of LKB1 at S431 downstream of ERK signaling in WT B cells.

Editor Comment 5: Show the requested staining for figure E4.

Response 5: Representative images of the requested flow cytometry staining to analyze NP+ GC B cells in WT-YFP and BKO-YFP mice have been added to revised Figure E4B.

Editor Comment 6: In addition, please go through the study one more time and ensure the claims made are supported by the data provided. Experimentally addressing some of the issues raised by referee 1 would be out of the scope of the present study, such as the elucidation of the cell extrinsic role of LKB1 or providing support for its role as a central regulator of T cell-dependent humoral immunity, but please rephrase wherever necessary. Note also that we would not insist on the in vivo IL-6 blockade experiment.

Response 6: We have revised the text to make certain all of the claims are supported by the data, as requested, and appreciate the recognition that many of the studies requested by referee 1 are beyond the scope of the current study.

Finally, we have included 5 extra figures in the online version of the manuscript as an Expanded View section, with the rest of the data now submitted as supplementary information. Therefore, we have re-formatted the original Figures E1, E2, E3, E4, and E6 as Expanded Figures E1-E5 and the original E5, E6 (E6 has been split into 2 figures), and E7 as Supplementary Figures S1-S3.

Sincerest thanks for your expertise and guidance in handling our re-revised submission, Nonia.

Response to Referees:

Referee #1:

Comment 1: Title conclusion is not proven - showing reduction in IL6 in tissue culture MEFs when NFkB is restored is not the same as showing Tfh and GC formation in vivo.

Response 1: In BKO-YFP mice, there is an increase in IL-6 production from LKB1- B cells (Figure 4A and B) and an increase in TFH cells *in vivo*. We provide additional mechanistic evidence showing that in LKB1- MEFs the NF-κB pathway is aberrantly activated and inhibition of this pathway results in repression of *Il6* transcripts. This data is consistent with and supported active NF-κB signaling in LKB1-YFP+ B cells from BKO-YFP mice (Figure 5F). These data, along with previously published studies (Karnowski A et al. 2012 J Exp Med; Eto D et al. 2011 PLoS One), are all supportive and consistent with IL-6 production in LKB1- B cells inducing IL-21 production in T cells, resulting in TFH cell differentiation of activated CD4+T cells.

Comment 2: IL6 is not shown to promote Tfh cell differentiation and expansion to support a 100-fold increase in steady state GC in vivo.

Response 2: IL-6 produced by LKB1- BKO B cells (Figure 4A and B) induces *Il21* in CD4+ T cells in vitro (Figure 4C). Previously published reports, (Karnowski A et al. 2012 J Exp Med; Eto D et al. 2011 PLoS One), have established a role for these two cytokines in the differentiation of activated CD4+ T cells into TFH cells. As pointed out by both reviewers 1 and 2, our data is consistent with IL-6 driving *Il21* transcription *in vitro* and the revised text in the results, abstract, and figure legend sections has been modified to state this result only.

Comment 3: Blockade of secreted IL6 from BKO B cells does inhibit IL21 expression but is not shown to disfavour Tfh differentiation and expansion.

Response 3: As shown in Figure 4D, IL-6 blockade suppresses the induction of *Il21* specifically in T cell co-culture with BKO B cells. We have revised the text to state this

result only.

Comment 4: It remains unclear to me what the demonstrated cell extrinsic role for LKB1 in B cells is that controls Tfh cell differentiation and GC formation.

Response 4: Aberrant IL-6 production from LKB1- B cells induces *Il21* expression in CD4+ T cells (Figure 4C). Previously published reports (Karnowski A et al. 2012 J Exp Med; Eto D et al. 2011 PLoS One) has established a regulatory role for these two cytokines in TFH cell differentiation and GC formation. There are likely additional LKB1 dependent, B cell extrinsic factors that promote the GC phenotype seen in BKO-YFP mice, but we focused on the role for IL-6 and IL-21 in this study as major factors promoting TFH cell differentiation.

Comment 5: It has also not been shown that LKB1 is a central regulator of T cell dependent humoral immunity since the one TD response studied is apparently completely normal.

Response 5: BCR signaling (Figure 6) results in LKB1 phosphorylation on an inhibitory serine that has been connected to LKB1 inactivation (Zheng B et al. 2009 *Mol Cell*), which would also be the case when *Lkb1* is deleted. A T cell dependent immune response in BKO mice is similar to that seen in WT mice in terms of antigen-specific antibody secretion (Figure E4C), most likely due to the chimerism of the BKO-YFP mouse line. As shown in Figure E4B, while there is an NP-specific response in BKO mice, it is significantly reduced in BKO mice at day 10 post-immunization compared to WT control mice.

Comment 6: This is a very dense manuscript to read and the nomenclature chosen is cumbersome but I can't think of an alternative. I think the facts as listed are well supported by the data. I disagree with the conclusions that are drawn as, to me, they are significant over-reach as indicated above.

Response 6: We have edited the text to increase clarity and to make sure that claims made are strongly supported by the data. We thank the referee for pointing out which claims require modification, and this has been done throughout the text.

Comment 7: I would also suggest the argument against IL6 blocking in vivo is a bit spurious. The authors could, for example, clear all existing GC by anti-CD40L treatment and then begin treatment in one group with anti-IL6 and another with an isotype control. If they have done any kind of a time course of GC onset in the mice (have they?) then they will now how long the maximum is they have to wait. My guess would be 2-3 weeks. I doubt they need 90. I doubt they need 20. 10 would probably do as it is a +/- assay. But if they don't want to do it, fine by me.

Response 7: We appreciate the reviewer comments on potential experimental design, which we have not pursued.

Comment 8: Scales are often presented without any point of reference. SO for example, the induction of IL21 in the T cell cultures. Is that a lot, a little, equal to a Tfh? The amount of cytokine detected in E6A - same questions relative to LPS macrophage for example. As they are, it is very difficult to know the significance of these numbers. Clearly more than WT, but enough for the proposed purposes/outcomes?

Response 8: In the co-culture *in vitro* assay, we have added comparisons for the expression of *Il21* from BKO co-cultured T cells to that of BKO CD4+ T cells and sorted TFH cells from LCMV infected WT mice to provide a point of reference in Figure 4D. Similarly, for a subset of the elevated inflammatory cytokines in BKO compared to WT B cells, we compared cytokine secretion to both WT B cells stimulated with the pro-inflammatory stimulator LPS as well as to bone marrow derived macrophages stimulated with LPS and we have added this analysis to revised Figure S2B, as suggested.

Comment 9: I would encourage the authors to show the stainings for GC and NP+GC+

cells in E4. This is important but there is no data presented other than the summary.

Response 9: Representative flow data for this analysis has been added to Figure E4B, as suggested.

Comment 10: Discussion cold be shorter, given issues outlined above.

Response 10: Because the paper is intricate, the discussion is needed to clarify and context the results and identifies logical next steps in this study progression.

Referee #2:

Comment 1: The authors argue based on the data in figure 2 that BKO GC B cells are undergoing a remarkable 10-fold more apoptosis than control (GC) B cells. Despite this, the BKO mice have at least 10-fold more GC B cells. Without seeing the purity of the GC B cells isolated from the two types of mice I remain unconvinced that the BKO cells have higher apoptosis. Rather, given the much greater frequency of GC B cells in the DKO mice and thus the much improved ability to purity them (they are almost non-existent in the control mice making it very unclear how they are really purified), taken together with the well established fact that GC B cells die extremely rapidly in vitro, it seems much more likely that the difference observed reflects a difference in the amount of 'real' GC B cells vs other (follicular etc) B cells in the 'purified' samples that are being compared. Other approaches would be needed if the authors really want to make this point such as comparing apoptotic cell frequencies in BKO and control mice with equivalently sized GCs by in situ TUNEL staining (or by FACS if mice with equivalent total GC responses can be generated).

Response 1: To assess apoptosis in Figure 2E, freshly isolated spleen cells were stained for markers of specific B cell subsets along with staining for active caspase 3 and analyzed by flow cytometry. No B cells subpopulations were isolated for this analysis and B cells were not cultured in vitro for any period of time.
To provide a second, independent method to evaluate BKO GC B cells for apoptosis, littermate control (LMC) mice were immunized with the T cell-dependent antigen, NP-CGG, and on Day 10 splenic GC B cells were assessed for active caspase 3 by flow cytometry without subpopulation purification or in vitro culturing. BKO mice showed a ~6-fold more B220+GL-7+ active caspase3+ cells by flow cytometry than day 10 NP-CGG immunized LMC mice from similarly sized GC B cell populations, indicating that BKO GC B cells are more apoptotic than WT B cells induced by a specific T cell-dependent antigen. This data has been added as Figure 2F, while the previous Figure 2F has been moved to Figure E3H.

Comment 2: It is stated in the abstract and in the results and figure 4 legend that BKO B cells induce more 'Tfh cell differentiation' based on an in vitro assay. Production of Tfh cells in vitro remains a 'holy grail' of the Tfh field and thus is not a remark to be taken 'lightly'. For a T cell to be termed a Tfh it minimally needs to be shown to have high expression of Bcl6 and at least some unique marker of this cell subset such as CXCR5. IL21 expression is not considered sufficiently Tfh restricted. The authors can state that BKO B cells can induce IL21-secreting T cell differentiation but they should not state they they can induce Tfh cell differentiation based on the in vitro data.

Response 2: We appreciate the reviewer comment and agree. The *in vitro* assay shows induction of *Il21* transcription and not full TFH cell differentiation. The abstract, results, and legend for Figure 4 have all been corrected to indicate this interpretation and conclusion.

Comment 3: The gene name of 36b4 and that it is a housekeeping gene should be stated in the methods.

Response 3: The gene name is *Rplp0* (60S acidic ribosomal protein P0). This has been added to the Materials and Methods section as part of the qRT-PCR methodology, as suggested.

Comment 4: In figure 6 it should be indicated how many blots are represented by the data shown. Is the second peak at 15 min reproducible?

Response 4: A revised legend for Figure 6 indicates that the image is representative of three independent western blots. There is a reproducible increase in S431 phospho-LKB1 between 10 and 20 mins of BCR stimulation.

3rd Editorial Decision 16 March 2015

I have now heard back from referee 2, who assessed your responses to both his/her and referee 1's concerns, and who now supports publication with no further comments. I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Many thanks again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

