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# c-Cbl regulates dendritic cell activation

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## Transaction Report:

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Editorial Decision 30 September 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. Please find enclosed comments from three scientists that evaluated conclusiveness and thus suitability of your study for our journal. All indicate interest in the study, but request much more convincing evidence that c-Cbl functions indeed directly as the executing p105 E3-ligase. Further, investigations into the mechanism of increased NF-kB activation in c-Cbl-/- DC would be important as this might explain increased cytokine production in these cells.

Conditioned on satisfactorily addressing these crucial points and responding appropriately to the essential concerns raised from our referees, we would be delighted to assess a thoroughly revised paper.

Yours sincerely,

Editor
The EMBO Journal

 

REFEREE REPORTS:

Referee #1:

NF-κB1 p105 is processed by the proteasome to produce the mature transcription factor p50. This process involves ubiquitination of p105 by an unknown E3 ligase. Based on the analysis of c-Cbl-/- DC and overexpression experiments in 293T cells, the authors propose that c-Cbl functions as the E3 ligase that controls p105 processing in TLR-stimulated DC.

The identification of the E3 ligase that regulates p105 processing is an important biological question. This study contains some data suggesting that this may involve c-Cbl, but fails to convincingly demonstrate that c-Cbl directly functions as p105 E3 ligase.

Specfic points

1. Figure 1A shows that c-Cbl deficiency alters TLR-induction of various cytokine proteins. These data should be analyzed statistically to determine whether detected differences are significant.

c-Cbl deficiency does not alter LPS induction of Il6, a gene which is positively regulated by p50 (1). If p105 processing is impaired in c-Cbl-/- DC, it would be expected that IL-6 induction would be reduced. What is the explanation for this discrepancy? Does c-Cbl deficiency affect LPS induction of TNF?

2. Evidence is presented to show that c-Cbl deficiency does not dramatically alter the phenotype of in vitro generated DC (Figure S1). However, to confirm that the changes in cytokine expression are actually to the absence of c-Cbl it is necessary to do a rescue experiment with retrovirally expressed c-Cbl.

3. A small increase in the fraction of CD4+ and CD8+ cells in the spleen is detected after transfer of Ova-pulsed c-Cbl-/- DC compared to wild type DC. It is unclear what this result is supposed to show, since it is not known if the increase in CD4+ and CD8+ T cells is antigen-specific, and could be a bystander effect. The number (and not fraction) of antigen-specific CD4+ and CD8+ T cells should be assayed (eg. by tetramer staining). In addition, it is essential to determine whether wild type and c-Cbl-/- DC are present in similar numbers in the chimeric mice, since it is possible that c-Cbl affect the survival of transferred DC. This needs to be checked for all similar DC transfer experiments.

4. The labeling of Figure 2 B and C appears to be incorrect from the figure legend. In Figure 2C (?), are the detected differences in cytotoxicity statistically significant?

5. It is claimed that there are 'reduced levels of TH2-driven IgG1 FROM Cbl-KO DC-primed mice' However, the LH graph in Figure 2E does not show this difference.

6. Figure S3A shows that Cbl-/- CD11c-enriched BMDC have slightly reduced LPS-induced ERK phosphorylation compared to wild type cells. As the difference in ERK phosphorylation is small, it should quantified (ERK-P/ERK-total) and tested for statistical significance. In addition, it is essential to show that JNK activation (which is TPL-2-independent) is unaffected by c-Cbl deficiency.

7. The quality of the TPL-2 blot in Figure 3A needs to be improved before it is possible to conclude that LPS-induced TPL-2 proteolysis is unaffected by c-Cbl deficiency. In addition, since it is well established that IKK-induced p105 proteolysis is required for TPL-2 activation, it is important to directly determine whether c-Cbl is required for TPL-2 activation by in vitro kinase assay. Also, the release of TPL-2 from p105, which is regulated by p105 proteolysis, should be determined by immunodepletion of p105 from cell lysates 2. These experiments will directly address the question of whether TPL-2 activation, which is dependent on IKK-induced p105 proteolysis, is regulated by c-Cbl.

8. In Figure 3A, is the increase in p50 after 2h stimulation of wild type levels simply due to

 

increased p105 expression? It is important to show p105 levels as well as p50, since Nfkb1 transcription is increased by LPS stimulation. The effect of c-Cbl deficiency on LPS stimulation on Nfkb1 mRNA levels should be determined by qRT-PCR.

9. If c-Cbl regulates p105 processing as suggested, why does LPS induce an apparent decrease in p105 levels in c-Cbl-/- cells that is similar to wild type cells (Figure S3B)? Also, why does MG132 treatment fail to block the LPS-induced decrease in p105 in c-Cbl-/- cells? The quality of the blots in this figure need to be improved.

10. Pulse / chase metabolic labeling experiments should be carried out to directly determine whether c-Cbl regulates p105 processing to p50 {plus minus} LPS.

11. c-Cbl deficiency reduces activation of AKT (Figure 3A). Does pharmacological inhibition of AKT activation with wortmannin and/or LY-294002 or AKT knockdown affect p105 processing to p50?

12. In Figure 3C, a blot showing p105 levels should be included.

13. In Figure 4A, quantitative RT-PCR should be used to assay the levels of IL-12 p35/p40 and IL-10. These data should be presented together with the protein data in Figure 1, at the beginning of the manuscript.

14. c-Cbl deficiency is shown to increase activation of an NF-κB reporter in LPS-stimulated DC (Figure 4C). The authors need to assay NF-κB directly by NF-κB EMSA + antibody supershifting, and investigate the mechanism underlying this effect (Rel subunit expression, IKK activation, Rel nuclear translocation). It is also interesting to determine whether the effect of c-Cbl deficiency is restricted to NF-κB activation. Does c-Cbl regulate the activation of AP-1?

The authors do not establish why proinflammatory cytokine gene expression is increased in c-Cbl-/- DC. The effects of c-Cbl deficiency on IL-12 induction do not appear to be consistent with the known effects of p50 deficiency on LPS-induced IL-12 expression in macrophages and DC 3, 4. This may be due to a compensatory increase in the activation of other NF-κB subunits as suggested by the NF-κB reporter data, or alternatively the regulation of other signaling pathways by c-Cbl. It is notable that the increase in IL-12 and decrease in IL-10 phenocopy Tpl2-/- DC stimulated with LPS 5.

15. In Figure 5 A and D, control blots should be included showing that each of the c-Cbl mutants were expressed at comparable levels. In addition, the detected differences should be analyzed statistically for significance. In Figure 5C, a blot showing p105 levels should be added.

What is the effect of wortmannin and/or LY-294002 on LPS-induced IL-12 p70 induction?

16. The published effects of p50 deficiency on IL-12p70 induction in DC and macrophages (see above) do not appear to be consistent with the inhibition of IL-12p70 production by c-Cbl-/- DC when transfected with p50 expression vector (Figure 5B). This may arise from the supra-physiological expression of p50 after transient transfection. How does the expression of p50 in c-Cbl-/- DC when transfected with p50 compare to wild type cells?

17. The levels of p105 and p50 in the experiments shown in Figure 6 A and B should be quantified. (The starting levels of p105 are clearly different between the wild type and ΔRING c-Cbl transfected cells.) By scanning several similar experiments, it will be possible to determine whether the proposed differences in the induction of p50 (relative to p105) in cells expressing wild type and ΔRING c-Cbl are statistically significant.

In Figure 6A, why do p105 levels decrease after CD40 cross-linking in cells expressing ΔRING c-Cbl if ubiquitination of p105 is blocked (Figure 6B)? Is this decrease due to constitutive p105 proteolysis and is it blocked by addition of MG132?

18. Several earlier studies have demonstrated that IKK-induced p105 proteolysis involves SCFβ-TrCP-mediated ubiquitination of p105 6-8. The authors propose that c-Cbl induces p105 processing

 

by binding to the same IKK-phosphorylated residues in the PEST region (Figure 6D). However, the possibility that c-Cbl regulates p105 processing via SCFβ-TrCP is not ruled out. This should be investigated by siRNA knockdown of SCFβ-TrCP. In addition, to confirm that c-Cbl is a direct E3 ligase for p105, it is necessary to analyze p105 ubiquitination and processing in vitro using purified proteins.

19. The effect of Fyn knockdown or inhibition with a small molecule inhibitor should be tested on LPS induced p50 production in DC.

20. The differences shown in Figure 7E should be analyzed statistically for significance.

References

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Referee #2:

In the present manuscript Chiou et al. provide evidence that c-Cbl is a negative regulator of TLR4-induced cytokine production in DC. Mechanistically, inducible processing of the NFKB1 precursor p105 to p50 is compromised in c-Cbl deficient DCs. As generation of p50 homodimers has been shown to decrease transcriptional activation of NF-kappaB target genes, decreased processing is suggested to be the primary cause for augmented cytokine production in c-Cbl KO cells. By showing that the E3 ligase activity of c-Cbl is enhancing p50 generation, the authors suggest that c-Cbl is the missing E3 ligase that is responsible for p105 precursor processing at least in DCs. As previous results have shown that SCF-betaTRCP is the ligase only involved in constitutive processing of p105, this study is a very interesting report, because it identifies c-Cbl as an E3 ligase for inducible p105 processing. However, some issues should be addressed.

The conclusion that diminished p50 production is the primary cause for enhanced IL-12 production in DCs is largely based on Fig. 5B, where it is shown that expression of p50 in Cbl-KO cells reduces IL12 production just like expression of c-Cbl. It would be interesting to see in how far this is a

specific effect of p50, e.g. by overexpressing p65 to see that this does not decrease and maybe even increase IL-12 expression. In addition, it would be good to analyze the expression of other cytokines, e.g. IL-10, IL-6 and IL-1beta, in this setting. This would support the conclusion that the effect of c-Cbl dependent p50 production is not restricted to IL-12 induction.

The conclusion that c-Cbl is the p105 E3 ligase controlling inducible processing is based on overexpression (Fig. 7). Differences in CD40 triggered ubiquitination in Fig. 6B are relatively weak. Is this a mono-ubiquitination? What is the size of the band? Additional evidence should be obtained to strengthen the assumption that c-Cbl is the E3 ligase for p105. The authors could determine endogenous p105 ubiquitination in wt and c-Cbl KO cells. Or by in vitro ubiquitination assay the authors could compare Cbl induced ubiquitination of WT-p105 or the 3A-p105 mutant that does not bind c-Cbl.

Specific points:

Fig. 1B: Something about the gating and the cell numbers is not matching. The very minor population of IL-12 gated cells after CpG are said to be almost 20% of the cells, whereas the much larger population after LPS induction represent only 14% of the cells. Are these numbers correct? Also the gates are different in every panel.

Fig. 2: Legend for 2B and C have been mixed up. IFNg and IL-4 should be mentioned in the Figure. In the text the authors state to Fig. 2E: '...concomitantly reduced levels of TH2-driven IgG1 from Cbl KO ...'. Is this slight reduction after 3 and 4 weeks significant?

Fig, 3A: p58/TPL2 is cut in a way that it is hard to see anything. Increased p50 DNA binding should be shown, e.g. by EMSA.

Fig. 4 A and B: The author's state: '...consistent with the observation that unstimulated Cbl-KO or Cbl-KD DCs expressed high levels of IL-12 and IL-6 (Figure 4B and 1A, respectively).' Whereas constitutively enhanced IL-6 production is evident in 1A, there is no effect on IL12 levels in unstimulated cells. Also not in Fig. 1E. Also IL-10 mRNA is strongly reduced in Fig. 4A, but the effects on IL-10 protein is marginal (Fig. 1A).

Fig. S4A: Why is the double mutant deltaRING/Y737F still binding to PI3Kp85alpha? If this goes through Y373, binding should be lost. Fig. S4C and A have been mixed up in the text. The name deltaRING for a point mutant is confusing.

Fig. 5: A: Expression controls should be shown. B: More target genes should be determined after p50 rescue (see above). C: What is the effect of Y369F on p50? This is important, because the Y369F mutant is an important control in Figure 7.

Fig. 6: B: Differences in ubiquitination are not convincing, also not in S5A. Is this mono-ubiquitination? At what size does it migrate? More data on c-Cbl dependent ubiquitination pf p105 are required (see above). D: Why is HA instead of p50 on the same blot as p105. Why is it not shown as in Fig. 6A?

Referee #3:

In the reviewed manuscript entitled "Essential role of c-Cbl in inducible p105 processing and dendritic cell activation", Chiou et al. identify a novel function of c-Cbl in activation-dependent processing of the p105. Previously known involvement of c-Cbl in TCr signaling, in TNF-induced apoptosis, and in regulation of FcγRII receptor in neutrophils implies that c-Cbl could also be involved in DC activation. Furthermore, it appears that the identity of the ubiquitin-ligase involved in degradation of C-terminus of p105 is not known, which provides additional rationale for the study.
To reveal the inhibitory role of c-Cbl in NF-kB-mediated signaling, the authors show that targeting of c-Cbl results in upregulation of pro-inflammatory cytokines and biases Th1 polarization in the format of antigen-presentation and vaccination (Figures 1 and 2). Analysis of intracellular LPS-

initiated signaling in BMDCs showed most of the components not affected except the ones that linked to p105. Finally, in search of the regulator of c-Cbl activity, they identified that Fyn is the kinase that activates c-Cbl and is positioned upstream of c-Cbl.
The study is carefully designed and performed and the overall impression of this work is quite positive, in particular support of the targeting data with silencing in vitro both experiments independently showing up-regulation of IL-12 in c-Cbl-deficient cells (Figure 3B) and add-back experiment confirming the effect of the knockout (Suppl. figure 3).
However, the following questions have to be addressed:

- Constitutive secretion of IL-6 by non-activated DCs (Figure 1A) looks very unusual, particularly given the fact that IL-6 is a second wave cytokine, which requires chromatin remodeling and new protein synthesis and therefore should not be produced in quiescent cells; quite strikingly the levels of IL-6 in non-activated cells are high thus making necessary to discuss in detail this observation and provide more thoughtful explanation;

-The legend to Figure 2B does not describe cytotoxicity test (as stated in the manuscript), in addition, B and C panels appear to be switched; in general, the figure legends have to be less cryptic; some sentences in the text such as "c-Cbl can inhibit proinflammatory cytokine secretion through LPS-induced p50" (page 8) have to be re-phrased as it is not quite clear what is happening with p50 and why is it induced by LPS; similar examples can be found elsewhere in the text;

- Supplementary Figure 3B needs loading control to be substituted on something more acceptable;

-In Figure 4, it is better to present the data by means of quantitative real-time PCR than gel- bands as the former has higher sensitivity and will help reconciling the mRNA data with the protein data (Figure 1A) which show attenuated levels of IL-10 whereas in Figure 4A IL-10 mRNA is depleted.
- Figure 6, panels A and D, needs better explanation of the time-points as it is not entirely clear where is the inducer added; also Figure 6D - 3A mutant of p105 - it is hard to notice the difference since the intensity of triple-A-p105 is less than the wild-type; with respect to the latter, authors should seriously consider using pulse-chase experiment instead of just transfection as tracking down the labeled p105 into p50 is far more conclusive; in fact, the pulse- chase was traditionally used in resolving a longstanding issue of co-translational versus post- translational production of p50 from p105.

Revision – authors' response 30 March 2011

**Referee #1:**

NF-κB1 p105 is processed by the proteasome to produce the mature transcription factor p50. This process involves ubiquitination of p105 by an unknown E3 ligase. Based on the analysis of c-Cbl-/- DC and overexpression experiments in 293T cells, the authors propose that c-Cbl functions as the E3 ligase that controls p105 processing in TLR-stimulated DC.

The identification of the E3 ligase that regulates p105 processing is an important biological question. This study contains some data suggesting that this may involve c-Cbl, but fails to convincingly demonstrate that c-Cbl directly functions as p105 E3 ligase.

Specific points 1. Figure 1A shows that c-Cbl deficiency alters TLR-induction of various cytokine proteins. These data should be analyzed statistically to determine whether detected differences are significant.

**Answer:** The revised data with statistical results can be found in the revised figure (**Fig. 1A**).

c-Cbl deficiency does not alter LPS induction of Il6, a gene which is positively regulated by p50[1]. If p105 processing is impaired in c-Cbl-/- DC, it would be expected that IL-6 induction would be reduced. What is the explanation for this discrepancy? Does c-Cbl deficiency affect LPS induction of TNF?

**Answer:** The cited report by Yamamoto *et al.* used the NF-κB1-deficient mouse line generated by Baltimore and colleagues (Sha et al., 1995), which is deprived of all p50-containing NF-κB protein complexes, including the purported p50 homodimer-IκBξ complex. According to the authors, selective p50 deficiency results in the abrogation of LPS-induced Il-6 expression in macrophages. In addition to their findings, p50 homodimers have also been reported to bind another IκB family member, Bcl-3, which negatively regulates many proinflammatory genes following TLR engagement (Carmody et al., 2007).

In Cbl-KO DCs, p50 levels remains relatively stable following LPS treatment **(Fig. 3A, panel 7**), though not completely ablated as it appeared to be in NF-κB1-deficient cells. Therefore, this might still enable the formation of stimulatory IκBξ-p50-p50 complex while at the same time, c-Cbl deficiency removes the effect of LPS-induced accumulation of more p50, which might otherwise facilitate the formation of inhibitory homodimer complexes (Heissmeyer et al., 1999). The inhibitory nature of accumulated p50 is further demonstrated by our new result in which ectopic expression of p50 in Cbl-KO DCs inhibits LPS-induced IL-12p70 and TNF-α, but not IL-6, implying the preferential formation of inhibitory p50 complexes at those promoters (**Fig. S4E**).

In addition, tissue specificity might be another possible explanation for this discrepancy. DCs derived from the same p50-deficient mice have been shown to express unaltered levels of LPS-induced IL-6 in a different report (Wang et al., 2007). In contrast, Yamamoto *et al.* have based all their observations on macrophages and MEFs (Yamamoto et al., 2004). This suggests the possibility that the observed effect of IκBξ on the p50 homodimer function might be restricted to macrophages and other cell types, but not DCs. On the other hand, the inhibitory role of p50 homodimers when paired with Bcl-3 has been observed in both DCs and macrophages, consistent with broader applicability (Carmody et al., 2007; Wessells et al., 2004). Our new results (not included in the original submission) suggest that c-Cbl deficiency upregulates LPS-induced TNF-α by DCs (**Fig. S4F**).

2. Evidence is presented to show that c-Cbl deficiency does not dramatically alter the phenotype of in vitro generated DC (Figure S1). However, to confirm that the changes in cytokine expression are actually to the absence of c-Cbl it is necessary to do a rescue experiment with retrovirally expressed c-Cbl.

**Answer:** We have indeed tried to use two independent viral systems to transduce BMDCs to achieve ectopic expression of c-Cbl, and neither of these systems could attain higher efficiency protein expression than that provided by Lonza "nucleofection". Therefore, we used nucleofection instead for the rescue experiment, and the results can be found in **Fig. S3F, S4E**, and all panels of **Fig. 5**, which suggest that re-

expression of WT c-Cbl in Cbl-KO DCs recapitulates the inhibitory effect of WT c-Cbl on cytokine secretion.

3. A small increase in the fraction of CD4+ and CD8+ cells in the spleen is detected after transfer of Ova-pulsed c-Cbl-/- DC compared to wild type DC. It is unclear what this result is supposed to show, since it is not known if the increase in CD4+ and CD8+ T cells is antigen-specific, and could be a bystander effect. The number (and not fraction) of antigen-specific CD4+ and CD8+ T cells should be assayed (eg. by tetramer staining). In addition, it is essential to determine whether wild type and c-Cbl-/- DC are present in similar numbers in the chimeric mice, since it is possible that c-Cbl affect the survival of transferred DC. This needs to be checked for all similar DC transfer experiments.

**Answer:** The increase in both CD4+ and CD8+ populations in the spleens of Cbl-KO DC-vaccinated mice was presented as total cell numbers (**Fig. 2A**). However, these results do not quantify the antigen-specific elements within the pool, as suggested. Therefore, we measured the level of antigen-specific response in the spleen by quantifying *in vitro* peptide-stimulated IFN-γ production (ELISpot) in both CD4+ and CD8+ splenocytes (**Fig. 2C**). In addition, we have also conducted SIINFEKL-MHC I $K^b$-PE tetramer staining on peripheral blood mononuclear cells (PBMCs) isolated from vaccinated mice (**Fig. S2F**). Consistent with the ELISpot data, mice vaccinated with Cbl-KO DCs have significantly higher levels of SIINFEKL-specific CD8 T cells (**Fig. S2F**).

We have also conducted a new assay for quantifying *in vivo* survival of vaccinated DCs. Our new results suggest that c-Cbl deficiency does not significantly influence the survival of adoptively transferred DCs *in vivo* (**Fig. S2G**).

4. The labeling of Figure 2B and C appears to be incorrect from the figure legend. In Figure 2C (?), are the detected differences in cytotoxicity statistically significant?

**Answer:** The corrected legends for **Fig. 2B and C** can be found in the revised manuscript. The difference between CblKO-E.G7 and WT-E.G7 groups is statistically significant ($P < 0.05$, **Fig. 2B**).

5. It is claimed that there are 'reduced levels of TH2-driven IgG1 FROM Cbl-KO DC-primed mice' However, the LH graph in Figure 2E does not show this difference.

**Answer:** This was indeed misinterpreted in our previous manuscript version. Accordingly, a correction was made to the interpretation of the **Fig. 2E** IgG1 ELISA result (revised manuscript).

6. Figure S3A shows that Cbl-/- CD11c-enriched BMDC have slightly reduced LPS-induced ERK phosphorylation compared to wild type cells. As the difference in ERK phosphorylation is small, it should quantified (ERK-P/ERK-total) and tested for statistical significance. In addition, it is essential to show that JNK activation (which is TPL-2-independent) is unaffected by c-Cbl deficiency.

**Answer:** We have conducted statistical analysis (not included in the previous manuscript) on the densitometric signals of ERK phosphorylation in LPS-stimulated, CD11c-enriched DCs. Our results suggest a significant difference at the 10-min time point (**Fig. S3A, right panel**). Consistent with our original manuscript, JNK phosphorylation is not influenced by c-Cbl deficiency.

7. The quality of the TPL-2 blot in Figure 3A needs to be improved before it is possible to conclude that LPS-induced TPL-2 proteolysis is unaffected by c-Cbl deficiency. In addition, since it is well established that IKK-induced p105 proteolysis is required for TPL-2 activation, it is important to directly determine whether c-Cbl is required for TPL-2 activation by in vitro kinase assay. Also, the release of TPL-2 from p105, which is regulated by p105 proteolysis, should be determined by immunodepletion of p105 from cell lysates[2]. These experiments will directly address the question of whether TPL-2 activation, which is dependent on IKK-induced p105 proteolysis, is regulated by c-Cbl.

**Answer:** Due to our initial concern for space, the Tpl-2 data was presented in a confounding manner in the previous manuscript. In order to present the results with greater clarity, we have re-cropped the Tpl-2

figures from the original blot and moved them to the supplemental figures with expanded explanation (**Fig. S3B**). Our data suggest that there is no discernable difference in both Tpl-2 isoforms between the WT and Cbl-KO group following LPS stimulation (**Fig. S3B**). Additionally, our results regarding the phosphorylation of IKKβ, shown to cause p105 phosphorylation and its degradation, also suggest that c-Cbl deficiency does not noticeably alter its intensity (**Fig. 3A, panel 1**). Together with our new results concerning total p105 levels (**Fig. 3A, panel 6**), we have concluded that the Tpl-2 pathway is NOT greatly affected by c-Cbl deficiency and not a key factor in the effects on DCs we describe. Therefore, we would argue that this obviates the need to conduct the *in vitro* kinase assay on Tpl-2 enzymatic activity.

8. In Figure 3A, is the increase in p50 after 2h stimulation of wild type levels simply due to increased p105 expression? It is important to show p105 levels as well as p50, since Nfkb1 transcription is increased by LPS stimulation. The effect of c-Cbl deficiency on LPS stimulation on Nfkb1 mRNA levels should be determined by qRT-PCR.

**Answer:** The p105 level turned out to be one of the most crucial aspects to this story. The original model posits that c-Cbl directly catalyzes induced p105 processing into p50. However, our new pulse-chase data challenge this hypothesis (**Fig. 6A**). Instead, we found that c-Cbl might enhance LPS-induced p50 accumulation by stabilizing p105 (total p105 level in revised **Fig. 3A, panel 6, and S3C**). In addition, this effect is dependent on the RING domain function of c-Cbl (**Fig. 5C**). However, it is still not completely clear how c-Cbl stabilizes p105 through its RING function, therefore, we have discussed the possibilities in the revised manuscript (Discussion).

It is also likely that increased p50 in WT DCs can be attributed at least partly to enhanced *Nfkb1* mRNA production following TLR engagement. Based on this hypothesis, the observed effect of c-Cbl deficiency on LPS-induced p50 levels might result from lower *Nfkb1* mRNA levels. If this hypothesis holds, it predicts that c-Cbl deficiency would reduce *Nfkb1* mRNA production following LPS stimulation.

However, our qRT-PCR result suggests that c-Cbl deficiency does not change the mRNA level of p105 regardless of the stimulation, therefore refuting this hypothesis (**Fig. S4H**).

9. If c-Cbl regulates p105 processing as suggested, why does LPS induce an apparent decrease in p105 levels in c-Cbl-/- cells that is similar to wild type cells (Figure S3B)? Also, why does MG132 treatment fail to block the LPS-induced decrease in p105 in c-Cbl-/- cells? The quality of the blots in this figure needs to be improved.

**Answer:** As mentioned in our answer to the previous point, the requested experiments have led us to adopt a different model to explain how c-Cbl facilitates p50 accumulation following TLR engagement. Accordingly, it explains why in Cbl-KO DCs, LPS induced p105 turnover as that observed in the WT DCs. In fact, our revised data show that the turnover of p105 in Cbl-KO DCs is even more pronounced than in WT counterparts, which suggests that c-Cbl stabilizes p105 under stimulatory conditions (**Fig. 3A and S3C**).

The experiment we conducted to show MG132-mediated inhibition of LPS-induced p105 turnover in Cbl-KO DCs suggests a delayed onset of LPS-induced degradation (**Fig. S4G**). This might be due to the relative insensitivity of the machinery to optimal MG132 treatment (10 μM for two hours), which drives p105 turnover. However, a similar effect was observed in our new data wherein we treated WT DCs with the same amount of MG132 for 2 hours (**Fig. S3D**). It is not clear to us why this is the case; however, it is possible that at later time points (2 hours following stimulation) p105 might be degraded in a proteasome-independent manner.

10. Pulse / chase metabolic labeling experiments should be carried out to directly determine whether c-Cbl regulates p105 processing to p50 ± LPS.

**Answer:** We have conducted the pulse-chase metabolic labeling experiment as requested multiple times and included our data in the revised manuscript (**Fig. 6A and S5A**). However, due to the poor quality of the blots generated by using the anti-p105/p50 antibodies currently available, we used an anti-HA anti-

body to immunoprecipitate protein products derived from an N-terminally tagged p105 construct transfected, along with other plasmids, into 293T cells. The new results have led to a revision of our original model, which explains how c-Cbl facilitates p50 accumulation (revised manuscript). The old model postulates that c-Cbl facilitates p50 accumulation by directly catalyzing p105 processing, while the revised model suggests c-Cbl does so by stabilizing p105.

11. c-Cbl deficiency reduces activation of AKT (Figure 3A). Does pharmacological inhibition of AKT activation with wortmannin and/or LY-294002 or AKT knockdown affect p105 processing to p50?

**Answer:** In order to uncouple the effect of Akt from other PI3K effector molecules, we chose to use Akt inhibitor II (Akti) (a substrate competitive PI analog), instead of a pan-PI3K inhibitor. Our results suggest that Akti does not change LPS-induced p105 processing and p50 accumulation (**Fig. S4A, left panel**). As a positive control to show the effect of Akti treatment, Akt inhibition by Akti led to increased IL-12 production (**Fig. S4A, right panel**), which is consistent with the proposed role of endogenous Akt (Androulidaki et al., 2009). Based on these observations, we have concluded that the lack of LPS-induced p50 accumulation in Cbl-KO DCs does not result from reduced Akt phosphorylation/activation.

12. In Figure 3C, a blot showing p105 levels should be included.

**Answer:** The results of a new knockdown experiment with p105 levels are included in revised **Fig. 3C**.

13. In Figure 4A, quantitative RT-PCR should be used to assay the levels of IL-12 p35/p40 and IL-10. These data should be presented together with the protein data in Figure 1, at the beginning of the manuscript.

**Answer:** The new qRT-PCR results are included as requested and can be found in the revised manuscript (**Fig. 1F**).

14. c-Cbl deficiency is shown to increase activation of an NF-κB reporter in LPS-stimulated DC (Figure 4C). The authors need to assay NF-κB directly by NF-κB EMSA + antibody supershifting, and investigate the mechanism underlying this effect (Rel subunit expression, IKK activation, Rel nuclear translocation). It is also interesting to determine whether the effect of c-Cbl deficiency is restricted to NF-κB activation. Does c-Cbl regulate the activation of AP-1?

**Answer:** We have conducted **several** different assays to clarify the effect of c-Cbl deficiency on NF-κB in DCs. We have quantified the nuclear translocation of p65, p50, and c-Rel. We have also conducted EMSA and a chromatin immunoprecipitation assay (ChIP assay) for the quantification of NF-κB binding to specific κB sites. Out data suggests that compared to the other NF-κB family members, nuclear translocation of p50 at the two-hour time point is significantly reduced in c-Cbl-deficient DCs (**Fig. S3G**). In addition, both our EMSA and ChIP results suggest that c-Cbl ablation enhances binding of NF-κB complexes, including the stimulatory c-Rel-p50 heterodimer, to different κB sites in DCs (**Fig. 4D, 4E, and 4F**), consistent with the observation of increased proinflammatory cytokines in Cbl-KO/KD DCs. The increased c-Rel binding to the κB sites might not result from enhanced nuclear translocation, since ablation of c-Cbl does not affect c-Rel translocation following LPS treatment (**Fig. S3G**). Importantly, our new data phenocopy Bcl-3-deficient macrophages (Carmody et al., 2007). According to Carmody *et al*., Bcl-3 deficiency destabilizes p50, leading to enhanced c-Rel loading onto NF-κB sites and an upregulation of proinflammatory cytokines, including IL-1β and TNF-α, which are also increased in Cbl-KO DCs (Carmody et al., 2007). In addition, destabilization of p50 in Bcl-3-deficient macrophages significantly altered the dynamics of NF-κB complex formation. Similarly, c-Cbl deficiency also causes reduction of p50 accumulation following LPS stimulation, which is accompanied by an alteration of the dynamics of NF-κB complexes, leading to enhanced c-Rel binding and the upregulation of multiple proinflammatory cytokines.

Finally, our data suggest that c-Cbl deficiency does not significantly affect AP-1 activity following TLR engagement, which is consistent with our western blot results of ERK and JNK phosphorylation (**Fig. 4A, right panel**). Therefore, our overall data suggest that c-Cbl's effect is mainly restricted to NF-κB.

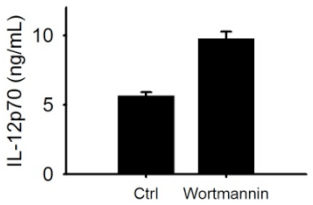
The authors do not establish why proinflammatory cytokine gene expression is increased in c-Cbl-/- DC. The effects of c-Cbl deficiency on IL-12 induction do not appear to be consistent with the known effects of p50 deficiency on LPS-induced IL-12 expression in macrophages and DC[3, 4]. This may be due to a compensatory increase in the activation of other NF-κB subunits as suggested by the NF-κB reporter data, or alternatively the regulation of other signaling pathways by c-Cbl. It is notable that the increase in IL-12 and decrease in IL-10 phenocopy Tpl2-/- DC stimulated with LPS[5].

**Answer:** As mentioned previously, we offered several rationales/reasons to explain this contradiction. In addition, given our new qRT-PCR results, we concluded that c-Cbl deficiency does not significantly alter IL-10 expression levels. Consistently, c-Cbl ablation in BMDCs also does not significantly alter their ability to induce a Th2-associated IgG1 antibody response after vaccination into naïve mice (**Fig. 2E, left panel**). Therefore, it is possible that the phenotype we have observed while using c-Cbl-deficient DCs is, rather than a Th1 bias along with a concomitant Th2 reduction, the result of an upregulation of a Th1-biased adaptive immune response.

15. In Figure 5 A and D, control blots should be included showing that each of the c-Cbl mutants were expressed at comparable levels. In addition, the detected differences should be analyzed statistically for significance. In Figure 5C, a blot showing p105 levels should be added.

**Answer:** New blots are included in the revised manuscript (**Fig. 5A and D**). Additionally, statistic analyses are included as suggested (**Fig. 5A and D**). A new p105 blot is added as suggested into **Fig. 5C**.

What is the effect of wortmannin and/or LY-294002 on LPS-induced IL-12 p70 induction?




**Answer:** Interestingly, we found that inhibition of the PI3K pathway by wortmannin enhanced LPS-induced IL-12p70 production by DCs (right), which is consistent with previous reports (Fukao et al., 2002).

16. The published effects of p50 deficiency on IL-12p70 induction in DC and macrophages (see above) do not appear to be consistent with the inhibition of IL-12p70 production by c-Cbl-/- DC when transfected with p50 expression vector (Figure 5B). This may arise from the supra-physiological expression of p50 after transient transfection. How does the expression of p50 in c-Cbl-/- DC when transfected with p50 compare to wild type cells?

**Answer:** The functions of p50 homodimers are contextual, depending on its binding partner (e.g., the IκB family member and co-activator, IκBξ, or the corepressor, Bcl-3), the complexes are expected to function very differently. In addition, p50 also forms heterodimers with c-Rel or p65/RelA, which are critical to the expression of many proinflammatory cytokines. Therefore, it is expected that the expression of some proinflammatory cytokines would be abrogated in the p50-deficient mice. However, as described above, the expression of p50 in c-Cbl-deficient DCs is not completely removed, therefore allowing the formation of some p50-containing complexes, including the p50 homodimer-IκBξ complex.

With regards to the result of **Fig. 5B**, the p50 level seen in Cbl-KO DCs transfected with the p50 construct is a bit higher than that expressed in the Cbl-KO DCs reconstituted with WT c-Cbl (**Fig. 5B**). The level of p50 in this assay is inversely correlated with the level of IL-12 induced, which supports our hypothesis.

17. The levels of p105 and p50 in the experiments shown in Figure 6 A and B should be quantified. (The starting levels of p105 are clearly different between the wild type and mRING c-Cbl transfected cells.) By scanning several similar experiments, it will be possible to determine whether the proposed differences in the induction of p50 (relative to p105) in cells expressing wild type and mRING c-Cbl are statistically significant.

**Answer:** Indeed, the starting levels of p105 are different between the two groups. This is secondary to the stabilizing effect of WT c-Cbl on the p105 protein level, which is also observed in DCs (**Fig. S7A, left panel**). This effect cannot result from enhanced p105 transcription because in 293T cells, the transfected p105 construct in both groups are identical. Additionally, this is certainly not caused by unequal loading because the expression of c-Cbl at all data points is quite similar. Finally, it is also noteworthy that this c-Cbl-mediated stabilizing effect on p105 depends on c-Cbl's RING domain function (**Fig. S5C**).

Due to the change of the current hypothesis, we chose to replace the original **Fig. 6A** data with the new pulse-chase result (**Fig. 6A and S5A**). Our new data suggest that c-Cbl deficiency (or disruption of the c-Cbl RING function) leads to reduced accumulation of p50 because of lower levels of synthesized p105 being processed, rather than because of reduced p105 processing into p50. The residual level of CID-induced p50 accumulation in the c-Cbl (RING)-deficient group observed with the pulse-chase experiment (**Fig. 6A and S5A**) could not be easily detected in Cbl-KO DCs (**Fig. 3A**), which might be due to the higher sensitivity that radioactive labels provide. However, the new western blots reveal a striking correlation between p105 and p50 protein levels regardless of the effect different c-Cbl mutants impose, further supporting our new model (**Fig. 5C, lower panel**).

The densitometric result of the original **Fig. 6B** (**Fig. 6E** in revised manuscript) was actually included in the original manuscript, which was represented by the ratio of the densitometric measurements of NTA-precipitated c-myc versus whole cell lysate-derived myc measurements (**Fig. 6E**).

17b. In Figure 6A, why do p105 levels decrease after CD40 cross-linking in cells expressing mutant RING c-Cbl if ubiquitination of p105 is blocked (Figure 6B)? Is this decrease due to constitutive p105 proteolysis and is it blocked by addition of MG132?

**Answer:** In the reconstruction model assay for p105 processing (in 293T cells), protein translation is blocked by cycloheximide treatment, which terminates both constitutive processing of p105 (Lin et al., 1998) and production of nascent p105 (which is coupled with constitutive processing or the biogenesis of p50 under steady state conditions). Under such circumstances, properly folded p105 can follow only two routes, either protein turnover or processing into p50 following stimulation (induced processing). Similarly in the pulse-chase experiment (**Fig. 6A**), the effect of *de novo* protein synthesis is removed.

In fact, the original **Fig. 6B** data suggests that there still exists a constitutive level of p105 ubiquitylation in the reconstruction model (**Fig. 6E**). Additionally, **Fig. 6E** reveals a comparable background level of p105 ubiquitylation in both the WT and the RING mutant c-Cbl groups (**Lane 5 and 7**), which might be catalyzed by other endogenous E3 ligase(s) of p105 and hence independent of c-Cbl's RING function. The constitutive ubiquitylation of p105 might contribute to the steady turnover of p105 revealed in both sets of original data in **Fig. 6A**. On the other hand, in the new pulse-chase assays where p105 is transfected more efficiently (indicated by co-transfected GFP) and labeled separately, the effect of p105 turnover becomes much less obvious likely because overexpressed p105 could have surpassed the processing capacity of the protein recycling system (**Fig. 6A and S5A, left panel**).

The effect of c-Cbl-mediated ubiquitylation on p105 is still not completely clear to us (Discussion). With our available data, we suspected that this might, although somewhat counterintuitively, stabilize p105. However, at this point we cannot rule out other possibilities of how c-Cbl's RING function can stabilize p105 protein, for example, by inhibition of inhibitors, like other E3 ligase(s) (see Discussion). These questions await future studies for further clarification.

We did not use MG132 in the 293T-based experiments. However, our other results derived from DC-based experiments suggest that MG132 blocks both LPS-induced p105 turnover and accumulation of p50 (**Fig. S3D**).

18. Several earlier studies have demonstrated that IKK-induced p105 proteolysis involves $SCF^{\beta\text{-}TrCP}$-mediated ubiquitination of p105[6-8]. The authors propose that c-Cbl induces p105 processing by binding to the same IKK-phosphorylated residues in the PEST region (Figure 6D). However, the possibility that c-Cbl regulates p105 processing via $SCF^{\beta\text{-}TrCP}$ is not ruled out. This should be investigated by siRNA

knockdown of $SCF^{\beta\text{-}TrCP}$. In addition, to confirm that c-Cbl is a direct E3 ligase for p105, it is necessary to analyze p105 ubiquitination and processing in vitro using purified proteins.

**Answer:** In order to ablate endogenous βTrCP function, we designed an siRNA sequence found in both βTrCP1 and βTrCP2 transcripts, because previous studies suggest functional redundancy shared by these paralogs (Guardavaccaro et al., 2003). Our data suggest a satisfactory level of βTrCP suppression induced by this siRNA (**Fig. S5D**). Consistent with previous studies, the results also suggest that total p105 levels are predominantly determined by the function of the E3 ligase $SCF^{\beta TrCP}$ in DCs in response to stimulation, because knockdown of endogenous βTrCP in DCs significantly inhibits LPS-induced turnover of p105 (**Fig. S5D and S5E**). On the other hand, this targeting does not lead to inhibition of LPS-induced p50 accumulation (**Fig. S5D and S5E**). Therefore, we conclude that $SCF^{\beta TrCP}$ does not play an important role in the regulation of LPS-induced p50 accumulation in DCs.

We have tried aggressively to develop an *in vitro* Ub assay with c-Cbl and p105 using several commercially available E2 ligases and have admittedly failed to generate convincing data to directly support our hypothesis that c-Cbl acts as a direct E3 ubiquitin ligase for p105 processing into p50. However, we did see autoubiquitylation of the immunoprecipitated WT c-Cbl (but not the mRING c-Cbl), suggesting that the *in vitro* assay was technically functional. Nevertheless, we would like to argue that the reason the ubiquitylation of p105 by c-Cbl cannot be reconstructed in a cell-free system is possibly because the interaction between the two proteins requires yet-unidentified binding partner(s). Accordingly, we have chosen to rephrase (i.e., “soften”) our statement in the revised manuscript with regards to the current hypothesis.

19. The effect of Fyn knockdown or inhibition with a small molecule inhibitor should be tested on LPS induced p50 production in DC.

**Answer:** Yes, our new study suggests that Fyn knockdown by RNAi reduces LPS-induced p50 accumulation significantly (**Fig. 7D**).

20. The differences shown in Figure 7E should be analyzed statistically for significance.

**Answer:** Statistical analysis was conducted as requested (**Fig. 7E**).

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**Referee #2:**

In the present manuscript Chiou et al. provide evidence that c-Cbl is a negative regulator of TLR4-induced cytokine production in DC. Mechanistically, inducible processing of the NFKB1 precursor p105 to p50 is compromised in c-Cbl deficient DCs. As generation of p50 homodimers has been shown to decrease transcriptional activation of NF-κB target genes, decreased processing is suggested to be the primary cause for augmented cytokine production in c-Cbl KO cells. By showing that the E3 ligase activity of c-Cbl is enhancing p50 generation, the authors suggest that c-Cbl is the missing E3 ligase that is responsible for p105 precursor processing at least in DCs. As previous results have shown that SCF-βTRCP is the ligase only involved in constitutive processing of p105, this study is a very interesting report, because it identifies c-Cbl as an E3 ligase for inducible p105 processing. However, some issues should be addressed.

The conclusion that diminished p50 production is the primary cause for enhanced IL-12 production in DCs is largely based on Fig. 5B, where it is shown that expression of p50 in Cbl-KO cells reduces IL12 production just like expression of c-Cbl. It would be interesting to see in how far this is a specific effect of p50, e.g. by overexpressing p65 to see that this does not decrease and maybe even increase IL-12 expression. In addition, it would be good to analyze the expression of other cytokines, e.g. IL-10, IL-6 and IL-1beta, in this setting. This would support the conclusion that the effect of c-Cbl dependent p50 production is not restricted to IL-12 induction.

**Answer:** We have conducted a new experiment regarding the effects of ectopic expression of both p50 and p65/RelA on the production of IL-12p70, IL-6, and TNF-α following LPS stimulation (**Fig. S4E**). As the results indicate, forced expression of p50, but not p65, in Cbl-KO DCs by nucleofection reduces LPS-stimulated TNF-α and IL-12 (**Fig. S4E**). However, expression of p50 does not inhibit IL-6 production induced by LPS (**Fig. S4E**). Importantly, similar effect was observed with regard to that of WT c-Cbl, which also inhibits LPS-induced IL-12p70 (**Fig. 1**) and TNF-α (**Fig. S4F**), but not IL-6 (**Fig. 1A**). In conclusion, our new observation further supports the result in **Fig. 5B** and our hypothesis, which suggests that c-Cbl inhibits LPS-stimulated production of proinflammatory cytokines through induction of p50 accumulation.

The conclusion that c-Cbl is the p105 E3 ligase controlling inducible processing is based on overexpression (Fig. 7). Differences in CD40 triggered ubiquitination in Fig. 6B are relatively weak. Is this a mono-ubiquitination? What is the size of the band? Additional evidence should be obtained to strengthen the assumption that c-Cbl is the E3 ligase for p105. The authors could determine endogenous p105 ubiquitination in wt and c-Cbl KO cells. Or by in vitro ubiquitination assay the authors could compare Cbl induced ubiquitination of WT-p105 or the 3A-p105 mutant that does not bind c-Cbl.

**Answer:** The effect of the enzymatic activity of c-Cbl on the ubiquitylation level of p105, measured in the current setting, was indeed weak, unless a constitutively active Fyn was co-transfected. We have tried to look at the endogenous ubiquitylation of p105 in DCs, as well as to set up an *in vitro*, cell-free system to directly address the effect of c-Cbl's function on p105 ubiquitylation. Unfortunately, both assays failed to offer satisfactory results, as described above. However, we have observed strong signals of c-Cbl autoubiquitylation in the *in vitro* Ub assay, suggesting that the assay itself was working technically. Although we cannot offer this direct evidence to support our original hypothesis, there is now a great deal of new and old data to support our new model (**Fig. 7F**).

For example, the major concerns of the current model, which concludes that c-Cbl catalyzes induced p105 processing, are 1) possible involvement of $SCF^{\beta TrCP}$ in the processing and 2) the possibility of c-Cbl enhancing p50 accumulation through processing-independent mechanism(s) (e.g., through enhancement of p105 stability). In the revised manuscript, we have included the results refuting the first possibility, which demonstrates that LPS-induced p50 accumulation, but not the p105 turnover, is insensitive to βTrCP ablation in DCs (**Fig. S5D and S5E**).

To our surprise, our new pulse-chase data supported the latter possibility (**Fig. 6A and S5A**). Altogether, our data currently suggest that the RING domain function of c-Cbl is required for its stabilizing effect on p105, which, according to our revised model (**Fig. 7F**), leads to enhanced p50 accumulation (revised manuscript). However, it is still unclear how c-Cbl's RING function stabilizes p105, especially following stimulation (**Fig. S5C**). We have discussed possible mechanisms in the Discussion of the revised manuscript.

The status of p105 ubiquitylation and its migration in the current model is discussed in detail in the following sections.

**Specific points:**

Fig. 1B: Something about the gating and the cell numbers is not matching. The very minor population of IL-12 gated cells after CpG are said to be almost 20% of the cells, whereas the much larger population after LPS induction represent only 14% of the cells. Are these numbers correct? Also the gates are different in every panel.

**Answer:** The inconsistency between the numbers, regarding cell percentages and the shapes of the contour figures, results from differences in thresholds set for individual groups with paired samples. Additionally, the reason some gates were set differently than others is due to a shift of the main body of cells that are within the non-IL-12-secreting population. Therefore, we chose to manually set individual gates in a manner such that they all lied precisely at the borders between marker positive and the large body of label negative events. However, we agree that this might be confusing and have therefore re-analyzed the data (**Fig. 1B and C**). The new data are presented as dot plots instead of contour plots, and all gates are identical. As stated above, there have been minor shifts within the main body of IL-12-negative cells in some of the samples. For example, the CpG-stimulated group and the re-analyzed data suggest a marginal downregulation of IL-12 in Cbl-KO DCs stimulated by CpG (**Fig. 1B and 1C**). This observation is consistent with our ELISA data, which suggest that CpG-induced IL-12 secretion in DCs is insensitive to c-Cbl ablation (**Fig. 1D**). Therefore, we have concluded that c-Cbl deficiency does NOT reproducibly alter TLR9-mediated signaling.

Fig. 2: Legend for 2B and C have been mixed up. IFN$\gamma$ and IL-4 should be mentioned in the Figure. In the text the authors state to Fig. 2E: '...concomitantly reduced levels of TH2-driven IgG1 from Cbl KO ...'. Is this slight reduction after 3 and 4 weeks significant?

**Answer:** We have reorganized the legend for Figure 2 in the revised manuscript. The stated difference with regard to IgG1 production in the immunized animals was incorrect. We have hence rephrased this statement in the revised manuscript.

Fig, 3A: p58/TPL2 is cut in a way that it is hard to see anything. Increased p50 DNA binding should be shown, e.g. by EMSA.

**Answer:** We have re-organized the Tpl-2 western results, and the revised data are now included amongst supplemental materials due to figure sizes (**Fig. S3B**). Our original data were presented inefficiently due to somewhat unequal migration between different lanes, and the cropped figure inevitably includes some nonspecific bands in the marginal lanes. Therefore, we have re-cropped the figures from the original blots and have presented them with clearer, wider margins (**Fig. S3B**).

We have also conducted several assays to clarify the changes of different NF-$\kappa$B family members. First, we have conducted western blot analyses to quantify the nuclear translocation of p50, p65/RelA, and c-Rel following LPS stimulation (**Fig. S3G**). Our data suggest that c-Cbl deficiency downregulates nuclear translocation of p50 at the two-hour time point, which is consistent with our result derived from the whole cell lysates (**Fig. 3A**).

Secondly, we have conducted EMSA and chromatin immunoprecipitation assays for the quantification of binding of different NF-κB family members to NF-κB sites. Our results from both assays consistently point to the upregulation of NF-κB recruitment, including c-Rel, to different κB sites by c-Cbl deficiency following LPS stimulation (**Fig. 4D, 4E, and 4F**). Enhanced c-Rel recruitment may lead to increased expression of proinflammatory cytokines, including IL-12, in c-Cbl-deficient DCs, given its critical role in their expression (Grumont et al., 2001; Wang et al., 2007). Consistently, prior reports also suggest that destabilization of p50 enhances the recruitment of stimulatory c-Rel-p50 or RelA-p50 heterodimers (Carmody et al., 2007). Therefore, our new data suggest that c-Cbl ablation leads to a change of NF-κB dynamics, including the upregulation of LPS-induced c-Rel-p50 binding, which is accompanied by a general reduction of p50 levels.

Fig. 4 A and B: The author's state: '...consistent with the observation that unstimulated Cbl-KO or Cbl-KD DCs expressed high levels of IL-12 and IL-6 (Figure 4B and 1A, respectively).' Whereas constitutively enhanced IL-6 production is evident in 1A, there is no effect on IL12 levels in unstimulated cells. Also not in Fig. 1E. Also IL-10 mRNA is strongly reduced in Fig. 4A, but the effects on IL-10 protein is marginal (Fig. 1A).

**Answer:** The levels of constitutive IL-12p70 expression presented in **Fig. 1A** (quantified by LINCOplex) and **Fig. 1E** are too low to be analyzed statistically with confidence. Therefore, we used ELISA assays to quantify the difference between WT and KO counterparts using supernatants of cells seeded at higher density, and the data are included in **Fig. 1G** of the revised manuscript.

We have conducted the real-time quantitative PCR analysis for IL-10 to further clarify the inconsistence between the protein and mRNA levels. Our new data suggest that c-Cbl ablation does not significantly affect its mRNA level (**Fig. 1F**), which supports the **Fig. 1A** result.

Fig. S4A: Why is the double mutant ΔRING/Y737F still binding to PI3Kp85α? If this goes through Y373, binding should be lost. Fig. S4C and A have been mixed up in the text. The name deltaRING for a point mutant is confusing.

**Answer:** There has been several reports describing the c-Cbl YXXM motif involved in PI3Kp85α recruitment (Miura et al., 2003; Standaert et al., 2004), and both the Y369 and Y737 residues of murine c-Cbl are located within this context. Therefore, it is reasonable to expect compensatory binding of PI3Kp85α after mutation of either one of the two residues (**Fig. S4B, lanes 5 and 6**). Consistent with this notion, replacement of both Y369/Y737 abrogates PI3Kp85α binding induced by CA-Src (**Fig. S4B, lane 7**). However, this does not fully explain why the single point mutation of Y737 to phenylalanine is sufficient to block CA-Src-induced PI3Kp85α recruitment (**Fig. S4B, lane 4**). Our interpretation of this observation is that Y737 under certain circumstances inhibits the c-Cbl RING function, which is abrogated upon RING mutation (**Fig. S4B, lanes 4 and 5**). In other words, it is possible that the reason we did not see the co-immunoprecipitation signal in lane 4 is that the recruited PI3Kp85α is degraded following c-Cbl interactions rather than not being recruited in the first place.

In the revised manuscript, statements made with regard to data presented in the original **Fig. S4A and C** are corrected.

We have re-named the ΔRING-c-Cbl mutant as mRING-c-Cbl (which stands for mutant RING c-Cbl) to better eschew obfuscation.

Fig. 5: A: Expression controls should be shown. B: More target genes should be determined after p50 rescue (see above). C: What is the effect of Y369F on p50? This is important, because the Y369F mutant is an important control in Figure 7.

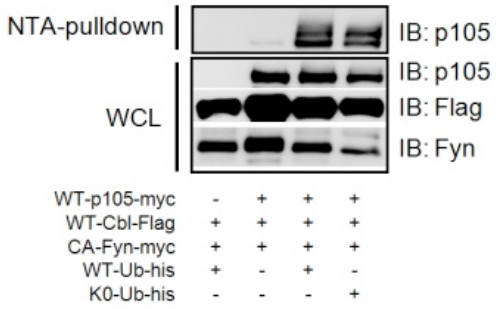
**Answer:** Expression controls are included for both **Fig. 5A and D**.

A new study has been conducted as described above with regards to the original **Fig. 5B** (**Fig. S4E**).

We have conducted additional nucleofection experiments to express the Y369F-c-Cbl mutant in Cbl-KO DCs (**Fig. S6B**). Our new data suggest that the Y369F mutant failed to induce p50 accumulation compared to the WT counterpart following LPS stimulation (**Fig. S6B, lane 2 and 6**). This observation further supports our model, suggesting that c-Cbl requires Y369 to be phosphorylated/activated by Fyn following stimulation.

Fig. 6: B: Differences in ubiquitination are not convincing, also not in S5A. Is this mono-ubiquitination? At what size does it migrate? More data on c-Cbl dependent ubiquitination of p105 are required (see above). D: Why is HA instead of p50 on the same blot as p105. Why is it not shown as in Fig. 6A?

**Answer:** Our new results utilizing the K0-Ub-his construct suggest that the ubiquitylation observed is possibly a mono-ubiquitylation on multiple lysine residues of p105 (right), which echoes the findings made by Kravtsova-Ivantsiv *et al* (Kravtsova-Ivantsiv et al., 2009). Ubiquitylated p105 migrates fairly closely with p105 from samples not transfected with the Ub constructs. As stated above, we have tried aggressively to develop an *in vitro* Ub assay to show a direct effect but failed to see this. However, we did see auto-ubiquitylation of the immunoprecipitated WT c-Cbl (but not the mRING c-Cbl), suggesting that the *in vitro* assay worked technically. Nevertheless, we would like to argue that the reason the ubiquitylation of p105 by c-Cbl cannot be reconstructed in a cell-free system is possibly because the interaction between the two proteins requires unidentified binding partner(s). However, since we have focused on a new and different aspect of this signaling pathway, the observations we made with regard to possible c-Cbl-mediated p105 ubiquitylation have become less crucial to the story. For these reasons, we have chosen to "soften" our statement about this possibility.




Due to the overall quality of the blot from which the original data of **Fig. 6D** derives, we chose to present p105 and p50 in separate figures in the original manuscript. We have replaced **Fig. 6D** data with a new figure, which includes both p105 and p50 within the same blot (**Fig. 6C**). Additionally, due to our revised focus on a new aspect of this pathway, we have drawn a revised conclusion from this experiment (revised manuscript).

**Referee #3:**

In the reviewed manuscript entitled "Essential role of c-Cbl in inducible p105 processing and dendritic cell activation", Chiou et al. identify a novel function of c-Cbl in activation-dependent processing of the p105. Previously known involvement of c-Cbl in TCR signaling, in TNF-induced apoptosis, and in regulation of FcγRII receptor in neutrophils implies that c-Cbl could also be involved in DC activation. Furthermore, it appears that the identity of the ubiquitin-ligase involved in degradation of C-terminus of p105 is not known, which provides additional rationale for the study.

To reveal the inhibitory role of c-Cbl in NF-κB-mediated signaling, the authors show that targeting of c-Cbl results in upregulation of pro-inflammatory cytokines and biases Th1 polarization in the format of antigen-presentation and vaccination (Figures 1 and 2). Analysis of intracellular LPS-initiated signaling in BMDCs showed most of the components not affected except the ones that linked to p105. Finally, in search of the regulator of c-Cbl activity, they identified that Fyn is the kinase that activates c-Cbl and is positioned up-stream of c-Cbl.

The study is carefully designed and performed and the overall impression of this work is quite positive, in particular support of the targeting data with silencing in vitro both experiments independently showing up-regulation of IL-12 in c-Cbl-deficient cells (Figure 3B) and add-back experiment confirming the effect of the knockout (Suppl. figure 3).

However, the following questions have to be addressed:

- Constitutive secretion of IL-6 by non-activated DCs (Figure 1A) looks very unusual, particularly given the fact that IL-6 is a second wave cytokine, which requires chromatin remodeling and new protein synthesis and therefore should not be produced in quiescent cells; quite strikingly the levels of IL-6 in non-activated cells are high thus making necessary to discuss in detail this observation and provide more thoughtful explanation.

**Answer:** Our revised data suggest that, in c-Cbl-deficient DCs, the dynamics of NF-κB family member proteins is reorganized, which facilitates the formation of a cellular state favoring pro-inflammatory cytokine induction. Consistent with this notion, our new study suggests that following stimulation, the recruitment of various NF-κB complexes to κB sites, including the c-Rel-p50 heterodimer, is enhanced under c-Cbl ablation, explaining the upregulation of proinflammatory cytokines under such circumstances. Additionally, it is also noted that in unstimulated DCs, c-Cbl ablation potentiates NF-κB recruitment to different NF-κB binding sites (**Fig. 4D and 4F, right panel**). Our result is similar to that reported by Carmody *et al.* where they found that Bcl-3 deficiency derepresses many proinflammatory cytokines. For example, they have shown that Bcl-3 ablation in macrophages destabilizes p50, which subsequently results in the reorganization of NF-κB complexes, including enhanced recruitment of both c-Rel and p65 to the κB sites (Carmody et al., 2007). Although we do not have the data to show the binding of NF-κB to the IL-6 promoter-localized NF-κB sites, it is possible that the recruitment of NF-κB dimers at this locus is similarly deregulated under steady-state condition in c-Cbl-ablated DCs.

- The legend to Figure 2B does not describe cytotoxicity test (as stated in the manuscript), in addition, B and C panels appear to be switched; in general, the figure legends have to be less cryptic; some sentences in the text such as "c-Cbl can inhibit proinflammatory cytokine secretion through LPS-induced p50" (page 8) have to be re-phrased as it is not quite clear what is happening with p50 and why is it induced by LPS; similar examples can be found elsewhere in the text;

**Answer:** The legends to Figure 2 have been corrected accordingly in the revised manuscript.

The accumulation of p50 following stimulation is largely derived from processing of p105, which is suggested by the requested pulse-chase data (**see below; Fig. 6A**). Additionally, this processing is largely dependent on proteasome function, as MG132 may block LPS-induced p50 accumulation in DCs (**Fig.**

**S3D**). The inhibitory nature of accumulated p50 was also investigated in our revised experiments, and we found that overexpression of p50, but not p65, in Cbl-KO DCs, downregulates LPS-induced IL-12p70 and TNF-α, without influencing IL-6 significantly (**Fig. S4E**). Importantly, the effect of ectopic p50 expression phenocopies that imposed by WT c-Cbl, including inhibition of LPS-induced IL-12p70 (**Fig. 1B-1E**) and TNF-α (**Fig. S4F**), but not IL-6 (**Fig. 1A**). Therefore, these observations further strengthen our current model wherein c-Cbl downregulates at least a subset of proinflammatory cytokines via p50 accumulation.

Of note, our findings are supported by reports of Conner *et al.* and Carmody *et al.*, wherein they have also found that accumulated p50 tends to play an inhibitory role on the induction of various proinflammatory cytokines (Carmody et al., 2007; Conner et al., 2010). Therefore, our current data strongly suggest that c-Cbl regulates induction of proinflammatory cytokines by facilitating p50 accumulation. However, due to the lack of direct evidence, we have accordingly chosen to soften our conclusion with regard to this aspect of our work (revised manuscript).

- Supplementary Figure 3B needs loading control to be substituted on something more acceptable;

**Answer:** The p105 data in the original **Fig. S3B** have been replaced with new results, providing better clarity (**Fig. 3A and S3C**).

- In Figure 4, it is better to present the data by means of quantitative real-time PCR than gel bands as the former has higher sensitivity and will help reconciling the mRNA data with the protein data (Figure 1A) which show attenuated levels of IL-10 whereas in Figure 4A IL-10 mRNA is depleted.

**Answer:** We have conducted the qRT-PCR assay for the quantification of IL-10 mRNAs extracted from paired samples and the result is included in revised **Fig. 1F**. Our data suggest that the induction of IL-10 seems insensitive to c-Cbl ablation, supporting the **Fig. 1A** data (**Fig. 1F and 1A, respectively**). Therefore, we have rephrased conclusions made with regard to the effect of c-Cbl ablation on IL-10 in the revised manuscript.

- Figure 6, panels A and D, needs better explanation of the time-points as it is not entirely clear where is the inducer added; also Figure 6D - 3A mutant of p105 - it is hard to notice the difference since the intensity of triple-A-p105 is less than the wild-type; with respect to the latter, authors should seriously consider using pulse-chase experiment instead of just transfection as tracking down the labeled p105 into p50 is far more conclusive; in fact, the pulse-chase was traditionally used in resolving a longstanding issue of co-translational versus post-translational production of p50 from p105.

**Answer:** Due to the adoption of a new model, we have replaced the original data (**Fig. 6A, original manuscript**) with the results of the requested pulse-chase experiment (**Fig. 6A**). The requested pulse-chase experiment was conducted by using our pathway reconstruction model in 293T cells, and this result has, in combination with other observations, largely led to a revision of the focus of our model which specifically explains how c-Cbl facilitates p50 accumulation. We have found that stimulation-induced p50 accumulation does indeed result from p105 processing in the current model system (**Fig. 6A**). In addition, we have found that, following LPS stimulation, p105 stability and p50 accumulation in DCs are both dependent on c-Cbl's RING function (**Fig. 5C**). We now hypothesize that this is because c-Cbl stabilizes p105 (**Fig. S3C**), especially following stimulation (**Fig. S5C**).

We have repeated the **Fig. 6D** experiment of the original manuscript and replaced it with the new data in the revised manuscript (**Fig. 6C**). In the revised figure, p105, p50, and iCD40T are presented within the same blot, facilitating a comparison between individual bands. The new result suggests that, in the presence of cycloheximide, expression of the 3A-p105 mutant is consistently lower than its WT counterpart even though the transfection is normalized (**Fig. 6C**). This can be explained by the observation that serine replacement within conserved DSGΨ motif of p105 disrupts its interaction with c-Cbl, which purportedly stabilizes it (**Fig. 6B**). Additionally, our new results suggest that c-Cbl's RING function is required to stabilize its interaction with p105 in response to stimulation (**Fig. S5C**). Therefore, we speculate that c-Cbl might stabilize p105 through its RING domain function, and disruption of the c-Cbl RING (**Fig. 6A**) or c-Cbl-p105 interaction (**Fig. 6C**) might eliminate this effect.

**Additional References:**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Editorial Decision 13 April 2011

Thank you very much for submitting your revisions on your work that reports contributions of c-Cbl to NF-kB signalling in the activation of dendritic cells. I did receive comments from two of the original referees that both appreciate the amount of experimental work aimed at clarifying the molecular mechanisms of c-Cbl. However, stepping back from the impressive amount of data, it becomes obvious that the actual molecular function(s) of c-Cbl in regulating p105 (via ubiquitination?) and in the control of p50 level/complex formation (to determine NF-kB transcriptional outputs) remain far from clear. Specifically, both scientists are in light of the EMSA- and pulse-chase results not convinced that alternative explanations have been sufficiently ruled out. With definitive molecular insight being one of the prime concerns for The EMBO Journal, I am really sorry to have to communicate that I do have no other choice than to decline further considerations of your study.

However, seeing the potential impact of the core message the study conveys, I did consult with the responsible editor of our sister journal EMBO reports that specializes in communicating important discoveries without fully explored mechanistic underpinnings.

Conditioned on shortening the paper (essentially leaving figures 6 and 7 as well as S5-7 out) and addressing the point that also p50-homodimers increase in the EMSA's upon c Cbl-depletion (as well as minor concerns regarding statistical analyses), they would in principle find the study suitable for EMBO reports. Your more focused revised version would be seen by one or two of the initial referees to ensure the concerns regarding the EMSA data have been adequately addressed. As an additional comment, I have been in contact with one of the referees that suggested attempting p50 ChIP upon c-Cbl knockdown as shown for other NF-kB-components in Fig. 4E.

I do hope that such a journal-transfer might be a valid and timely option for you and kindly ask you to get in touch with my colleague at EMBO reports. I also hope that despite the negative outcome on this occasion you still might consider our journal for publication of your future studies.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The authors have addressed some of my initial criticisms. However, they have substantially altered their model in the revised manuscript, and now propose that c-Cbl regulates p50 production by stabilizing p105 levels in unstimulated cells. Although c-Cbl is suggested to require its RING activity to mediate this function, the mechanism by which c-Cbl stabilizes p105 is not established. This significantly reduces the impact of the study. In addition, the authors now do not think that Cbl functions as an E3 ligase controlling p105 processing, which was one of the most novel aspects of the initial submission.

Specific points

1. OK.

2. OK.

3. OK.

4. OK.

5. OK.

 

6. In the graph show in Figure S3A, it is not indicated whether the difference in ERK phosphorylation in the CblKO cells is significant after 10min stimulation, although this is claimed in the rebuttal.

7. The authors state that the 'Tpl-2 pathway is not greatly affected by c-Cbl deficiency'. LPS activation of ERK in BMDC is known to be mediated by TPL-2, so why is ERK phosphorylation reduced in CblKO cells + LPS stimulation (Figure S3A).

8. The authors claim that Cbl stabilizes p105 based on the pulse-chase data shown in Figure 6A. However, the data appear to show that more p105 is produced during the 30min pulse in cells co-transfected with WT-Cbl compared to empty vector. Less p105 is also produced in the pulse of cells co-transfected with mRING-Cbl compared to WT-Cbl. These data imply that WT-Cbl increases p105 translation, since p105 mRNA levels are not altered. It does not indicate that WT-Cbl stabilizes p105 protein. Surprisingly, the graphical representation of the pulse-chase data does not include the empty vector control (Figure S5A). In addition, the effects of c-Cbl deficiency on p105 levels in BMDC are modest (Figures 3A). These results are presented graphically in Figure S3C. However, the data appear to be derived from a single experiment. Are the data normalized to a loading control? Are the detected differences statistically significant (analysis of multiple experiments required)? Also, the x-a
xis
of the graph is not labeled properly.

9. OK.

10. Not convinced by this model. See point 8.

11. OK.

12. OK.

13. Are the differences in IL-12p40, IL-10 and IL-12p35 mRNAs between WT and Cbl-KO cells statistically significant? (Figure 1F)

14. The authors propose that Cbl deficiency reduces p50 levels. However, the EMSA data in Figure 4A appear to show increased binding of p50 homodimers, as well as p50-RelA and p50-cRel heterodimers, in cells in which Cbl has been knockdown. What is the explanation for this discrepancy?

15. OK.

16. OK.

17. See point 8 about proposed function of Cbl in stabilizing p105. As noted in the summary above, the mechanism by which Cbl stabilizes p105 and the role of ubiquitination is not established.

18. Are the inhibitory effects of βTrCP knockdown on LPS-induced p105 proteolysis statistically significant? (Figure S5E)

19. OK.

20. OK.

Referee #2:

The authors have addressed all issues that I have raised in the first round of revision and the manuscript has considerably improved by these changes. In fact the new experiments that have been integrated have led to a different model. As the authors state, c-Cbl is apparently required for

 

maintaining p105 stability and accumulation of p50 rather than triggering p105 processing to p50. Even though a new mechanism is proposed to cause p50 accumulation, the basic concept that enhanced p50/p50 homodimer formation counteracts transcriptional activity exerted by p65/p50 or cRel/p50 heterodimers remains the same. Due to the changes made it becomes questionable, if this model is still valid. The authors need to consider the two following points:

1. The new EMSA experiments in Figure 4 are not really supporting the conclusion that c-Rel/p50 and p65/p50 binding is increased upon c-Cbl knock-down, while p50/p50 binding is decreased. In fact, the EMSA experiments suggest that also binding of p50 homodimers is increased after siRNA c-Cbl (Fig. 4D and F). It seems that overall NF-kB DNA binding activity is enhanced when c-Cbl amounts are reduced. Could there be a much simpler explanation for enhanced cytokine production in c-Cbl KO DC? It is well established that p105 acts as a cytosolic IkB that prevents activation of NF-kB heterodimers. Thus, reduced p105 levels in c-Cbl KO cells may simply enhance overall NF-kB activation due to increased amounts of cytosolic IkBs. At least this needs to be discussed.

2. The data on the ubiquitination of p105 by c-Cbl are still rudimentary and - as the authors mention themselves in the Discussion - it is completely unclear how c-Cbl triggered p105 ubiquitination relates to the enhanced stability of the protein. As stated, c-Cbl may destabilize a factor that is required for maintaining p105 stability. Overall, the role of p105 ubiquitination in this process remains completely elusive and therefore I don't see why it should be included in the paper. In fact this shortening could sharpen the conclusions.

Revision – authors' response 21 April 2011

We greatly appreciate your consideration of our manuscript.

The current study maintains that c-Cbl negatively regulates DC function by attenuating TLR-induced expression of multiple proinflammatory cytokines. Although full-length c-Cbl deficiency leads to significant alterations of multiple signaling pathways, our analysis of various c-Cbl mutant alleles suggests a strong correlation between the observed phenotype and p105/p50 proteins, in particular (Fig. 5). Additionally, evidence presented indicates that c-Cbl stabilizes p105 and its breakdown product, p50.

While the exact molecular mechanism is quite complex and still not entirely clear, our current data suggest that c-Cbl's inhibitory function hinges upon its stabilization of p105 and/or p50 protein(s). For example, we have noted that, upon ectopic expression of p50 into Cbl-KO DCs, p50 selectively attenuates cytokines that are also regulated by c-Cbl. However, even this model cannot fully explain our EMSA observations, wherein recruitment of all detected forms of NF-κB complexes at NF-κB sites, including p50 homodimers, is upregulated upon c-Cbl ablation (Fig. 4). Since p105 also functions as an IκB protein via its ankyrin repeats, reduction of p105 protein level can potentially release preexisting NF-κB dimers, including the p50 homodimers (Discussion). Consistent with this hypothesis, macrophages derived from the NF-κB1ΔC mice (wherein only the IκB-like function of p105 is knocked out while retaining p50) reveal a similar pattern of EMSA NF-κB binding (Ishikawa et al., 1998). Therefore, we cannot rule out the possibility that c-Cbl might inhibit proinflammatory cytokines by stabilizing, in addition to p50, the p105 protein.

With regards to changes made to the now shortened, more tightly written manuscript:

1 We have removed supplementary Fig. S3A, since we had based all our conclusions on non-enriched BMDCs with the exception of the CD11c column-enriched DCs used in Fig. S3A. Since column-enriched DCs may be partly activated, this may have provided unnecessarily confounding observations. One of our original reviewers for EMBO J had expressed concerns with regard to potential effects in the

 

Tpl2/ERK axis because of the observed effect of c-Cbl ablation on p105 protein level. However, we have consistently observed that in non-column-enriched BMDCs, c-Cbl deficiency does not alter the Tpl2-ERK pathway, even though the total p105 level is reduced. We suspect that this might be due to i) the relative insensitivity of Tpl2 to p105 reduction and/or ii) c-Cbl might only associate with and therefore stabilize p105 molecules that are not bound to Tpl2 (the "Different Pools" Hypothesis, Discussion).

2 The original Fig. S4A has moved to S4F and the rest of Fig. S4 adjusted accordingly, due to manuscript editing.

3 Figs. 6, 7, and S5-S7 have been removed, as requested. Their respective texts, in both Results and Discussion sections, have also been removed.

4 We have now focused our discussions on both the key observation of the deregulation of multiple cytokines upon c-Cbl ablation and have tried to offer possible models for the counterintuitive aspects of the EMSA data that doesn't parallel the total protein levels of various NF-κB complexes.

5 Minor statistical concerns were corrected.

6 Although one reviewer had requested we attempt an anti-p50-based CHIP assay in DCs to complement our c-Rel and RelB-based CHIP assays, we appreciate being waived this requirement. Not only would it require months to obtain enough c-Cbl KO DCs for this experiment, (the c-Cbl-KO line has become increasingly more difficult to breed to homozygosity over time in our and others hands), but also the p50 protein is part of multiple NF-kB complexes and it would be almost impossible to distinguish inhibitory from stimulatory p50 complexes by this approach. Further, all the c-Cbl-KO data we obtained was recapitulated using siRNA-nucleofected c-Cbl-reduced DCs. Of course, this method does not produce as many DCs as maturation of wildtype bone marrow precursors from mice.

Overall, this manuscript highlights a novel role for the E3-ligase, c-Cbl, in DC activation to add to well-established roles in T cell activation and other cells. The identification of the RING domain as the key to c-Cbl-mediated downregulation of pro-inflammatory cytokines should also help in the translation of these findings towards improved, more potent DC-based vaccines. We anticipate that this manuscript will be well-received by your readers.

Thank you again for your support.

Cited reference:
Ishikawa, H., Claudio, E., Dambach, D., Raventos-Suarez, C., Ryan, C. and Bravo, R. (1998) Chronic inflammation and susceptibility to bacterial infections in mice lacking the polypeptide (p)105 precursor (NF-kappaB1) but expressing p50. J Exp Med, 187, 985-996.

Editorial Decision 17 May 2011

I have now heard back from referee 2 of your previous submission to The EMBO Journal and I am happy to say that we would be happy to publish your study in EMBO reports, pending shortening and reformatting of the manuscript. You will find the referee report below, in which s/he also gives some suggestions on how to shorten the study.

As I mentioned in my previous email, the presentation of a merged results & discussion section - which we encourage- will aid in shortening, but the introduction and reference section will also have to be reduced. Basic materials and methods essential to the understanding of the experiments must be retained in the main body of the manuscript, but more detailed explanations necessary for the

 

reproduction of the experiments -including the sequence of probes and primers- should be included in supplementary information. Please also ensure that in the final ver4sion the number of experiments performed, definition of the error bars and information on statistical analysis is provided in the figures legend of the main text and supplementary information where appropriate.

I look forward to seeing a final version of your manuscript when it is ready. I will be out of the office at a meeting for the rest of the week, and will handle your manuscript as soon as possible upon my return.

I am happy to be the bearer of good news!

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

This is a highly interesting manuscript on the effects of cCbl on TLR signaling in DCs and especially on the regulation of the NF-kB system and the balance between p105 and p50 levels.

I think that the manuscript can be shortened in parts to more rigorously meet the length criteria of EMBO Rep. However, it is not possible to completely remove data, without significant loss of information. Some panels may be moved to the supplement (e.g. Fig. 1C, E and semi-quantitative PCR in F; Fig 2D, E; Fig 4 AP1 in A, supershift in D; F) to shorten the figures. The introduction and discussion could be condensed.

Specific point:

Figure 1A: Does N.S. stand for 'not significant'? This is confusing, because the results start immediately with the enhanced IL-12 production seen in the first panel of 1A. If this is due to the deviations obtained with pooled data sets from BMDCs, it at least requires some sort of comment.

Revision – authors' response 3 June 2011

As requested, we have further shortened our manuscript according to the guidance provided using great care to avoid loss of content. I believe we have succeeded in both goals, leaving a more tightly crafted, yet informative manuscript for your readers. The figures have also been converted to higher resolution TIFF files as required.

Thank you again for your support.

Editorial Decision 10 June 2011

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

 

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

Yours sincerely,

Editor
EMBO Reports

 