Dephosphorylation of Carma1 by PP2A negatively regulates T cell activation

Andrera C. Eitelhuber, Sebastian Warth, Gisela Schimmack, Michael Duwel, Kamyar Hadian, Katrin Demski, Wolfgang Beisker, Hisaaki Shinohara, Tomohiro Kurosaki, Vigo Heissmeyer and Daniel Krappmann

Corresponding author: Daniel Krappmann, Helmholtz Zentrum Muenchen -German Research Center for Environmental Health, Institute of Toxicology

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Thank you for submitting your manuscript to the EMBO Journal. Your study has now been been seen by three referees and their comments are provided below.

While the referees appreciate the described PP2A/Carma1 interaction, they also raise significant concerns with the analysis that I am afraid preclude its publication here at this stage. In addition to the technical concerns raised, the referees bring up 2 major issues. Firstly, they find that the present analysis does not provide strong enough data in support of that PP2A directly de-phosphorylates pSer-645 Carma1 and that this is what causes the enhanced IL-2 production, in particular considering the broad substrate specificity of PP2A. Secondly, as the PP2A/Carma1 interaction is constitutive, it remains unclear how Carma1 phosphorylation and de-phosphorylation is regulated upon T-cell stimulation e.g. how does initial T-cell stimulation lead to Carma1 phosphorylation and is the activity of PP2A regulated upon T-cell activation? Overall, given the concerns raised, I am afraid that I see not other choice, but to reject the manuscript at this stage.

However, given the potential interest in this work, I can offer that if you are able to strengthen the work considerably and address the concerns raised by inclusion of additional data that we are willing to look at a resubmission. I should point out that a resubmission is considered as a new submission; we consider the novelty of data at the time of resubmission and may, if needed, bring in new referee(s).

I thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be
more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This is an interesting manuscript in which Eitelhuber et al. identify PPP2R1A, in a 2-H screen, as a new Carma1 interacting protein. They use coIP experiments to try to show that PPP2R1A is recruited to the active CBM complex upon T activation. Experiments using siRNA attempt to show that the knockdown of PP2A enhances Ser645 phosphorylation of Carma1. They also show that this knockdown leads to increased activation of IL-2 and IFN-g production.

Although the finding that PP2A interacts with Carma1 is of interest, the quality of some critical experiments is not optimal, which makes it very difficult to judge whether the hypothesis is supported by the present experimental evidence or not (see below). However, the major problem of this paper is that as the interaction of PP2A is constitutive, the mechanism whereby the cell promotes its activation upon T cell stimulation is not clear at all. Also the use of PP2A knockdown is not adequate because PP2A is implicated in too many things and their experiment do not prove that the mechanism whereby PP2A is working in T cells can be ascribed solely to the control of Carma1 phosphorylation.

Specific Comments:
1. The EMSA of Fig. 2D is unacceptable. They should show a whole gel and identify by supershift experiments the components of the NF-kB complex(es). NFAT WB and shifts should also be shown. Although no effects are reported for Ca2+ entry, however, NFAT is a transcription factor regulated by phosphorylation/dephosphorylation by several kinases in a very complex manner, and it is still possible that the PP2A knockdown might affect its function. This must be tested.
2. The experiment of Fig. 2E is not adequate as they show the knockdown of PPP2R1A/B in EFs but the experiment is done in T cells (Fig. 2F). The knockdown must be shown in the same cell type in which the functional experiment is carried out.
3. The quality of the size-exclusion fractionation of Fig. 3A is not informative. And the experiment of Fig. 3B is not easy to understand: why are Bcl10 levels not shown if this is what it is being precipitated?
4. The WBs of Fig. 4 for the detection of PPP2R1A/B are of very low quality, inconsistent between the different panels (note that in some it is a simple band whereas in others, like in 4C, is a dirty smear).
5. Again, the quality of the blots in Fig. 5 is very poor. Particularly 5D and 5E are unacceptable. I do not see any phosphorylation of Carma1 at S645 in the siGFP section of Fig. 5D.

Referee #2 (Remarks to the Author):

The manuscript "Dephosphorylation of Carma1 by PP2A negatively regulates T cell activation" by Eitelhuber et al presented a series of interesting studies, suggesting that by binding to Carma1, PP2A regulatory subunit PPP2R1A recruits the catalytic subunit PPP2CA to the CBM complex and dephosphorylates Carma1, thereby, negatively regulating T cell activation. The authors identified PPP2R1A as a Carma1-binding partner through yeast-two-hybrid screen, which was further confirmed by Co-IP in HEK293 cells by transient transfection experiments. Fine mapping showed that PPP2R1A binding to the very C-terminal part of the Carma1 GUK domain. The authors observed that PPP2R1A depletion by siRNA knockdown enhanced IL-2 mRNA induction, NF-kB DNA binding in Jurkat cells (transfected with siRNA) when stimulated with CD3/28 or P/I, and

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resulted in more IL-2 and IFNgamma producing cells in primary CD4+ T cells (transduced with Adenoviruses carrying shRNA) when stimulated with CD3/28. By Co-IP, the authors showed that Carma1 constitutively associated with PPP2R1A, and recruited PPP2R1A to Bcell10 upon P/I stimulation in Jurkat T cells and in primary CD4+ T cells. Finally, the authors showed that PPP2R1A depletion by siRNA resulted in enhanced and prolonged Carma1 phosphorylation at Ser645, but not upstream PKC phosphorylation at Thr538, upon P/I stimulation in Jurkat Cells. Based on these observations, they concluded that PP2A negatively regulates T cell activation by dephosphorylating CARMA1 in T cells. Although their results are interesting and potentially important, it is premature to draw the conclusion, and additional experiments are needed.

PP2A has broad substrate specificity. PP2A has been found to dephosphorylate numerous substrates, including IKK complex, as well as MEKK3 that was recently shown to involve in TCR-induced signaling and NF-kB activation. It cannot rule out the possibility that the observed up-regulation of IL-2 production was due to the negative effect of PP2A on IKK and MEKK3. Therefore, the data presented in the manuscript is not sufficient to draw the conclusion.

It is necessary to perform a dephosphorylation assay to directly demonstrate that PP2A selectively dephosphorylates the phosphorylated-Ser645 in Carma1 in vitro, which may provide the direct evidence that PP2A dephosphorylates Ser645 Carma1.

Specific comments:

1. Authors argued that the NF-kB DNA binding is significantly higher with PPP2R1A depletion upon CD3/28 stimulation. However, Figure 2D showed that only slightly higher levels of NF-kB DNA binding were observed in knockdown cells compared to the mock cells. Therefore, this data is not convincing.

2. In Figure 2F, the knockdown effect in the primary T cells was not convincing. It is necessary to show the knockdown efficiency too.

3. In Fig. 3B, why Carma1 is not in Fraction 21-23 following PI stimulation? Majority of them should be in Fraction 21-23 based the result in Fig. 3A.

4. In Fig. 5B, 5D and 5E, the quality of these Western blotting for Carma1 P-S645 is low.

5. In Figure 5F, it is necessary to show the PPP2R1A/B levels.

Referee #3 (Remarks to the Author):

In this manuscript, Eitelhuber et al. identify the regulatory subunit of the protein phosphatase PP2A as a direct binding partner of Carma1 in a two-hybrid screen. Evidence is presented that Carma1 binds to the PP2A regulatory subunit PPP2R1A in 293T cells and T cells. Silencing of PP2AR1A expression enhances IL-2 and IFNgamma production in primary T cells and IL-2 production in Jurkat cells, and correlates with reduced phosphorylation of Carma1 at Ser 645, a previously identified site of activating phosphorylation by PKCtheta. Collectively, these findings suggest a negative regulatory role of PP2A in T-cell activation that might depend on Carma1 dephosphorylation.

The manuscript is of good technical quality and the reported findings are novel and highly interesting. Especially the effect of PP2A silencing on the formation of the Carma1-Bcell10 complex is highly convincing. Both the negative regulatory role of PP2A in TCR-induced NF-kB activation and Carma1 phosphorylation are convincingly demonstrated. However, in its present form, the manuscript does not provide sufficient evidence for a direct dephosphorylation of Carma1 by PP2A. The authors should address the following points.

Major points

1) The authors show that PPP2R1A binds to the GUK domain of Carma1. One prediction of this model is that a Carma1 mutant lacking the GUK domain shows increased/more persistent
phosphorylation at Ser 645. The authors should express such a construct in the Carma1-deficient cell line and assess the strength/persistence of its stimulation-induced phosphorylation.

2) The authors claim that PP2A directly dephosphorylates Carma1 on Ser 645, while not interfering with PKCtheta activation, but in its present form, the paper does not convincingly exclude this possibility, nor the possibility that other phosphorylation sites of Carma1, targeted by other Ser/Thr kinases, are affected. Data presented in Fig. 5F need to be strengthened by additional experimental evidence for a lack of effect of PP2A on PKCtheta, for example by in vitro kinase assays or by assessing autophosphorylation of PKCtheta on Thr 219. Moreover, the authors should show that other Carma1 phosphorylation events remain unaffected in the absence of PP2A.

3) It would be nice if some of the data obtained by PP2A silencing could be confirmed by an alternative approach. For example, okadaic acid has been shown in other studies to inhibit PP2A activity. Does pretreatment of T cells with okadaic acid lead to increased or more persistent Carma1 phosphorylation on Ser 645? Or can the authors use a dominant negative (catalytically inactive) version of PP2A to show an effect on Carma1 phosphorylation?

Minor points

1) Based on data presented in Fig. 4, the authors claim that PP2A binds to Carma1 already in unstimulated cells. However, the Carma1 IPs from Carma1 deficient cells (which serve as the most important negative controls) clearly reveal some non-specific binding of PP2A (Fig. 4E). Therefore, these data should be interpreted more carefully unless additional evidence for binding in unstimulated cells can be provided (for example by providing longer exposures of Fig. 4A and 4E or an additional, isotype-matched control for the Bcl10 IPs in Fig. 4B-D).

2) The levels of IL-2 secretion in Fig. 2B should be indicated in pg/ml. The observed stimulation-induced increases are surprisingly small when comparing to the fold changes in mRNA levels in Fig. 2C. How where the data normalized? Could there be a problem with normalization of the data (subtraction of background?).

Response to Referee #1

We are happy to see that the reviewer judges the manuscript interesting.

Although the finding that PP2A interacts with Carma1 is of interest, the quality of some critical experiments is not optimal, which makes it very difficult to judge whether the hypothesis is supported by the present experimental evidence or not (see below).

We have revised many experiments and are confident that our data are now of sufficient quality to support our findings (see belw).

However, the major problem of this paper is that as the interaction of PP2A is constitutive, the mechanism whereby the cell promotes its activation upon T cell stimulation is not clear at all.

We agree that the constitutive Carma1-PP2A interaction is puzzling at a first glance. However, that is what we see and we have now provided more data to strengthen this finding (new Figures 3A and B). We also include novel data to show that the upstream kinase PKCq is transiently recruited to Carma1 upon T cell activation (new Figures 4H and I). These data lend further support to our model that the activation of the CBM complex is initially induced by the recruitment and activation of PKCq. We can even show that PPP2R1A associated Carma1 is transiently hyper-phosphorylated after stimulation (Figure 3B), suggesting that the transient boost of local PKCq kinase activity on Carma1 probably shifts the balance towards phosphorylation. In contrast to PKCq, PP2A is constitutively associated with Carma1 and thus being able to rapidly eliminate the activating phosphorylation. Such a control by constitutively associated PP2A could help to assure that the CBM complex is disassembled rapidly after prolonged stimulation or when the antigen stimulus has
been removed. Furthermore, the constitutive interaction to Carma1 could also help to prevent CBM assembly and activation in the absence of antigen stimulation. We have changed part of the discussion (page 16-17) to make this point more clear.

Also the use of PP2A knockdown is not adequate because PP2A is implicated in too many things and their experiment do not prove that the mechanism whereby PP2A is working in T cells can be ascribed solely to the control of Carma1 phosphorylation.

We disagree with the assumption that PP2A knock-down is not adequate. Of course PP2A is implicated in many different processes. However, we provide evidence that transient knock-down of PP2A is not impairing all cellular functions. Calcium influx (Suppl. Fig. 5A), NF-AT activation (new Suppl. Fig. 5B), PKCα phosphorylation and activation (Fig. 4G and new Suppl. Fig. 7) is largely unaffected in PP2A knock-down cells. We have added siRNA against the PP2A catalytic subunit PPP2CA that shows highly similar effects when compared to siPPP2R1A (Figure 2B, 2G and 4E). We have also included new experiments using the PP2A inhibitor okadaic acid (new Figure 4F and 6D). We demonstrate that recombinant PPP2CA can dephosphorylate Carma1 in vitro (new Figure 5A). All these data support our assumption that PP2A is a Carma1 phosphatase. We are not claiming that the action of PP2A can be solely ascribed to the control Carma1 phosphorylation. Especially regarding the long term effects on IL-2 production and secretion after many hours of stimulation PP2A will certainly regulate other processes. In fact we have mentioned in the discussion (page 18) that PP2A was also shown to control nuclear activation of the IL-2 promoter by AP1 and CREB. However, by focusing our biochemical analysis on the critical initial events of T cells activation (Carma1 phosphorylation, CBM assembly, IKK/NF-κB activation) we can convincingly show that PP2A influences this early step of NF-κB signaling, which is absolutely crucial for optimal IL-2 production and T cell activation.

1. The EMSA of Fig. 2D is unacceptable. They should show a whole gel and identify by supershift experiments the components of the NF-κB complex(es). NFAT WB and shifts should also be shown. Although no effects are reported for Ca2+ entry, however, NFAT is a transcription factor regulated by phosphorylation/dephosphorylation by several kinases in a very complex manner, and it is still possible that the PP2A knockdown might affect its function. This must be tested.

We have repeated the EMSA and show now enhanced NF-κB DNA binding with three different siRNA targeting PP2A - two against PPP2R1A and one against PPP2CA (Figure 2G and Suppl. Fig. 4A). All siRNA show an enhancement of NF-κB DNA binding. Due to space constraints we only show part of the gel with NF-κB band shift in Figure 2G. We have now included a supershift analysis in Suppl. Fig. 4B to demonstrate that we are indeed detecting NF-κB/DNA complexes that predominantly contain p50/p65. For EMSA and supershift analysis in the supplement the entire gel including the free probe is shown.

By EMSA analysis we have now determined activation of the transcription factor NF-AT that is activated by the phosphatase calcineurin. Knock down of PPP2R1A or PPP2CA does not significantly influence the inducible DNA binding of NF-AT (Suppl. Fig. 5B).

2. The experiment of Fig. 2E is not adequate as they show the knockdown of PPP2R1A/B in EFs but the experiment is done in T cells (Fig. 2F). The knockdown must be shown in the same cell type in which the functional experiment is carried out.

By using adenoviral gene transfer, previous obstacles to achieve efficient knock-downs in primary mouse CD4 T cells have been overcome. To stress this point we have replaced the evaluation of PP2A knockdown by adenoviral shRNAs transduction of MEF cells with Western blots of FAC-sorted 10% highest GFP-expressing CD4 T cells (Figure 2D). The GFP positive cells were the cells that have been gated for in the single cell analysis of cytokine production (Figure 2E).

3. The quality of the size-exclusion fractionation of Fig. 3A is not informative. And the experiment of Fig. 3B is not easy to understand: why are Bcl10 levels not shown if this is what it is being precipitated?

We agree that the presentation of the experiment was somewhat misleading. We have revised the figure and integrated the Bcl10-IP into the old Figure 4 (now Figure 3C). The gel elution profile we have moved to the supplement (Suppl. Fig. 6). We have used three pools from the collected gel
filtration fractions. Pool I contains the high molecular weight CBM complex, pool II Carma1 and pool III preassembled Bcl10-Malt1. Efficient co-precipitation between Bcl10 and PPP2R1A is only seen in the high molecular weight pool I from stimulated cells, which also contain Carma1 (new Figure 3C). Together with Figure 3D that shows no co-precipitation of Bcl10 and PPP2R1A in Carma1 deficient T cells, these data provide compelling evidence that binding of PP2A to the CBM holo-complex in activated T cells is mediated by Carma1.

4. The WBs of Fig. 4 for the detection of PPP2R1A/B are of very low quality, inconsistent between the different panels (note that in some it is a simple band whereas in others, like in 4C, is a dirty smear).

We have repeated many of the experiments and added further controls to provide better WB (new Figure 3A, D and E). We also performed an anti-PPP2R1A IP to show constitutive interaction of endogenous PPP2R1A with Carma1 (new Figure 3B).

5. Again, the quality of the blots in Fig. 5 is very poor. Particularly 5D and 5E are unacceptable. I do not see any phosphorylation of Carma1 at S645 in the siGFP section of Fig. 5D.

The co-IPs after siRNA knock-down were usually done with fewer cells, because we needed to transfect a lot of cells to get to the same amounts. Under these conditions we were sometimes able to see clear Carma1 phosphorylation in PPP2R1A knock-down cells, even though it was barely detectable in control cells. We have repeated Figure 5D (now Figure 4D) using more cells and also include an experiment using siPPP2CA (new Figure 4E) and okadaic acid (new Figure 4F).

Response to Referee #2

We are pleased to see that the referee rates the manuscript as interesting and of potential importance.

PP2A has broad substrate specificity. PP2A has been found to dephosphorylate numerous substrates, including IKK complex, as well as MEKK3 that was recently shown to involve in TCR-induced signaling and NF-kB activation. It cannot rule out the possibility that the observed up-regulation of IL-2 production was due to the negative effect of PP2A on IKK and MEKK3. Therefore, the data presented in the manuscript is not sufficient to draw the conclusion.

It is true that PP2A has broad substrate specificity and was also suggested to target other substrates in the NF-kB pathway, including IKK complex components, MEKK3 or p65. We have mentioned this in the introduction and also taken this up in the discussion (page 17-18). Some older data are actually quite controversial, as for instance PP2A can activate or inactivate the IKK complex. MEKK3 controls IKK/NF-kB activation in T cells (Shinohara et al., 2009) and PP2A was recently shown to dephosphorylate Thr516 and Ser 520 of MEKK3 (Sun et al., 2010). However, it is unknown if these sites are phosphorylated upon T cell activation and whether this is influenced by PP2A. At present there are no commercial antibodies available to test this. Importantly, these putative PP2A targets (IKK, MEKK3 or p65) are all downstream of Carma1 and thus their phosphorylation and activity is expected to be influenced by the Carma1 phosphorylation status.

We can clearly demonstrate that the early and critical Carma1 Ser645 phosphorylation event is counteracted by PP2A. In agreement with this, PP2A inactivation promotes enhanced CBM complex formation which is well documented to be required for IKK/NF-kB activation and IL-2 production. Thus, we are providing a clear connection between a molecular target and a downstream effect.

We want to emphasize that we are not claiming that the observed upregulation of IL-2 is solely caused by Carma1 dephosphorylation. In fact we mention additional effects of AP1/CREB that have been published previously (page 18). However, on the level of Carma1 phosphorylation and the direct effects on the CBM complex and NF-kB signaling we are certain that our data are supporting the drawn conclusions. We have modified the Discussion on page 18 to make this point more clear.

It is necessary to perform a dephosphorylation assay to directly demonstrate that PP2A selectively de-phosphorylate the phosphorylated-Ser645 in Carma1 in vitro, which may provide the direct evidence that PP2A dephosphorylates Ser645 Carma1.
We now include in vitro dephosphorylation assays (new Figure 5). For this, we use the endogenous Carma1 after stimulation that is phosphorylated on Ser645 as a substrate for the catalytic subunit PPP2CA. We performed two types of assays. In the first assay we added recombinant PPP2CA which indeed removes the Carma1 Ser645 phosphorylation (Figure 5A). To see, if the catalytic activity of PPP2CA is required, we also transfected HA-PPP2CA wt or the catalytically inactive mutant HA-PPPCA (H118N; L199P). After CD3/CD28 stimulation, we immunoprecipitated in parallel HA-PPP2CA and Carma1 and incubated the precipitates. Under these conditions only wt, but not mt PPP2CA is able of removing S645 phosphorylation from Carma1 (Figure 5B).

1. Authors argued that the NF-kB DNA binding is significantly higher with PPP2R1A depletion upon CD3/28 stimulation. However, Figure 2D showed that only slightly higher levels of NF-kB DNA binding were observed in knockdown cells compared to the mock cells. Therefore, this data is not convincing.

We have repeated the experiment using the second siRNA against PPP2R1A as well as the siRNA against PPP2CA (new Figure 2G; Suppl. Fig. 4A/B). Please also see our response to comment 1 of referee#1. Even though the increase in NF-kB DNA binding is moderate, it is reproducibly seen with the different siRNAs targeting PP2A. In addition, we have determined IKK phosphorylation in PPP2R1A knock-down cells and we also see enhanced IKK phosphorylation when PP2A has been depleted (new Figure 2H), clearly indicating stronger NF-kB activity as a result of augmented upstream signaling.

2. In Figure 2F, the knockdown effect in the primary T cells was not convincing. It is necessary to show the knockdown efficiency too.

We would like to draw the reviewer’s attention to several new points that we now make:

First, by using adenoviral gene transfer, previous obstacles to achieve efficient knockdowns in primary mouse CD4 T cells have been overcome. To stress this point we have replaced the evaluation of PP2A knockdown by adenoviral shRNAs transduction of MEF cells with Western blots of FAC-sorted 10% highest GFP-expressing CD4 T cells (Figure 2D). We have mentioned this on page 7-8 in the Results.

Second, to increase the confidence in the cell intrinsic effect after knockdown of PP2A observed in primary T cells, we have included additional samples that we analyzed in a bar diagram (Figure 2F), which represent CD4 T cells that were infected and stimulated in parallel, but restimulated and analyzed 6 instead of 5 days after CD4 isolation. Our data now consistently show that the frequency of IL-2 and IFNγ positive cells increase between 1.5 -3 fold due to expression of an shRNA against PPP2R1A (see also page 8 in the Results).

Finally, we would like to point out that the extent of IL-2 accumulation in the supernatant of Jurkat T cells that were stimulated with PMA/Ionomycin or anti-CD3/CD28 ligation for ten or more hours (Figure 2B; Suppl. Fig. 3A) cannot be directly compared with an increased frequency of primary CD4 T cells, which make IL-2 protein during acute anti-CD3/CD28 stimulation. In addition to the difference in cell types and duration of stimulation, the ELISA measurement not only integrates IL-2 production due to cell activation but also cell proliferation. Nevertheless, we find that the 1.5-3 fold induction of IL-2 positive primary T cells upon PPP2R1A knockdown is relatively close to the 3-5 fold accumulation of secreted IL-2 that can be observed in Jurkat T cells with a PPP2R1A knockdown after PMA or CD3/CD28 stimulation.

3. In Fig. 3B, why Carma1 is not in Fraction 21-23 following PI stimulation? Majority of them should be in Fraction 21-23 based on the result in Fig. 3A.

There is no Carma1 detected in fractions 21-23, because this is a Bcl10-IP and preassembled Bcl10-Mal1 is shifted together with Carma1 into the high molecular weight fractions 11-14 (CBM complex fraction). Please also refer to Oeckinghaus et al., EMBO J. 2007, 26(22): 4634-4645 for further details. The presentation of the experiment has been misleading. We have revised the figure and combined it with the other co-IP experiments (new Figure 3C and Suppl. Fig. 6; also see response to comment 3 of referee#1). The combined gel filtration and Bcl10-IP clearly shows that
PPP2R1A is indeed associated with the CBM holo-complex and not with Carma1 containing sub-complexes in activated T cells.

4. In Fig. 5B, 5D and 5E, the quality of these Western blotting for Carma1 P-S645 is low.

We have revised Figure 5D (now 4D) with more cells after siRNA transfection to get clearer results. In addition we have included a new Figure 4E showing enhanced Carma1 S645 phosphorylation after PPP2CA knock-down. Please also see our response to comment 5 of referee#1. We do not agree with the low quality of Figure 5B (now 4B) that shows a peak of Ser645 phosphorylation already after 5-15 min, which is followed by a relatively fast decay. We would like to emphasize that the detection of S645 phosphorylated Carma1 is not trivial, because it comprises only a small pool of cellular Carma1 even after stimulation. However, all data clearly show enhanced Ser645 phosphorylation of Carma1 when PP2A has been down-regulated or inactivated.

5. In Figure 5F, it is necessary to show the PPP2R1A/B levels.

We have included this Blot.

Response to Referee #3

We are pleased that the referee highlights the good technical quality and novel and highly interesting findings.

However, in its present form, the manuscript does not provide sufficient evidence for a direct dephosphorylation of Carma1 by PP2A.

We have included new experiments that show that PPP2CA can act as a direct Carma1 phosphatase in vitro (see new Figure 5A and B; see below and response to referee#2).

Major points

1) The authors show that PPP2R1A binds to the GUK domain of Carma1. One prediction of this model is that a Carma1 mutant lacking the GUK domain shows increased/more persistent phosphorylation at Ser 645. The authors should express such a construct in the Carma1-deficient cell line and assess the strength/persistence of its stimulation-induced phosphorylation.

This is a good idea and we also cloned Carma1 deletion mutants to test it. Unfortunately, we found that the GUK and the very C-terminus of Carma1 that binds to PP2A is not only serving a negative regulatory function, but its deletion abolishes NF-kB activation (see Figure 4C). These results preclude an analysis of Carma1 phosphorylation status in PP2A binding mutants, because also initial activation is defective (see page 16 in the Discussion).

2) The authors claim that PP2A directly dephosphorylates Carma1 on Ser 645, while not interfering with PKCtheta activation, but in its present form, the paper does not convincingly exclude this possibility, nor the possibility that other phosphorylation sites of Carma1, targeted by other Ser/Thr kinases, are affected. Data presented in Fig. 5F need to be strengthened by additional experimental evidence for a lack of effect of PP2A on PKCtheta, for example by in vitro kinase assays or by assessing autophosphorylation of PKCtheta on Thr 219. Moreover, the authors should show that other Carma1 phosphorylation events remain unaffected in the absence of PP2A.

We now show direct dephosphorylation of Carma1 by PP2A in vitro (new Figure 5; see also response to referee#2). Since we have looked only at Thr538 phosphorylation of PKCq, the reviewer is right that PP2A could also regulate other phosphorylations, e.g. autophosphorylation of Thr219 or even others that have not yet been identified. We have included a PKCq kinase assay after PPP2R1A knock-down to show that CD3/CD28 induced PKCq activity is largely unaffected by PPP2R1A knock-down (Suppl. Fig. 7). We could not obtain a commercial antibody to test PKCq autophosphorylation on Thr219 after PP2A knock-down.
Concerning Carma1 we have tested two further Carma1 phospho-specific antibodies (against phosphor-Thr110 and phosphor-Ser555) that were raised against chicken Carma1 (Shinohara et al, J Ex Med 2006). The antibodies have been originally generated and tested for the detection of phospho-Carma1 in DT40 B cells from chicken. We were unable to detect phosphorylation at these residues in response to T cells activation. Either the antibodies do not cross-react with human Carma1 (despite considerable sequence identity) or Carma1 is not effectively phosphorylated at these sites in human T cells. Thus, we do not have the tools to perform the suggested experiment. However, we would like to emphasize that we were very careful with our interpretation of the results and we do not claim that PP2A is selectively influencing Ser645 phosphorylation of Carma1. Of course PP2A may also act on other positive phosphorylation events in Carma1 and this may even amplify the effect. We have modified the Discussion on page 16 to emphasize this point.

3) It would be nice if some of the data obtained by PP2A silencing could be confirmed by an alternative approach. For example, okadaic acid has been shown in other studies to inhibit PP2A activity. Does pretreatment of T cells with okadaic acid lead to increased or more persistent Carma1 phosphorylation on Ser 645? Or can the authors use a dominant negative (catalytically inactive) version of PP2A to show an effect on Carma1 phosphorylation?

We have now added experiments using the PP2A inhibitor OA to show that also pharmacologic inhibition led to increased Carma1 Ser645 phosphorylation and CBM complex formation after T cell activation (new Figure 4F and 6D). We have added this as additional evidence to show that not only downregulation, but also inhibition of catalytic activity has a similar effect. These data are supporting our model that PP2A acts as a Carma1 phosphatase.

We have used a catalytic inactive PPP2CA mutant as a control for Carma1 dephosphorylation in vitro. However, we were unable to see cellular effects on Carma1 phosphorylation using PPP2CA dominant negative mut or wt. Endogenous PP2A is an abundant protein and therefore dominant negative approaches are difficult. In addition, PPP2CA activity strictly requires the association with the PPP2R1A, which further complicates a dominant-negative approach using a single subunit.

Minor points

1) Based on data presented in Fig. 4, the authors claim that PP2A binds to Carma1 already in unstimulated cells. However, the Carma1 IPs from Carma1 deficient cells (which serve as the most important negative controls) clearly reveal some non-specific binding of PP2A (Fig. 4E). Therefore, these data should be interpreted more carefully unless additional evidence for binding in unstimulated cells can be provided (for example by providing longer exposures of Fig. 4A and 4E or an additional, isotype-matched control for the Bcl10 IPs in Fig. 4B-D).

We provide better experimental data for our observations that PPP2R1A is associated with Carma1 already in unstimulated T cells. We show Carma1 IP now as a comparison between Jurkat and JPM50.6 cells (Figure 3A). We do the reverse PPP2R1A IP including isotype and JPM50.6 controls (Figure 3B). We also revised the comparison of Bcl10 IP from JPM50.6 and Jurkat cells (Figure 3D).

Indeed, we detect a faint signal at the size of PPP2R1A in Jurkat and JPM50.6 cells after Bcl10 IP. Since we also detect a weak signal at the same size after Carma1 IP from Carma1-deficient JPM50.6 cells, we are quite sure that this band is just representing a background. Clearly, this signal is much weaker when compared to the association of PPP2R1A with Carma1 or the CBM complex in Jurkat T cells.

2) The levels of IL-2 secretion in Fig. 2B should be indicated in pg/ml. The observed stimulation-induced increases are surprisingly small when comparing to the fold changes in mRNA levels in Fig. 2C. How where the data normalized? Could there be a problem with normalization of the data (subtraction of background?).

We now indicate IL-2 levels in pg/ml. When reanalyzing the data we also noted a mistake in our previous calculation of relative IL-2 levels after P/I stimulation, because we did not use the correct the dilution factor. This at least partially explains the discrepancy between mRNA and protein amounts. We apologize for this error and thank the reviewer for the close look. There is still some discrepancy for CD3/CD28 stimulation, but we generally see that the Jurkat T cells are secreting
much less IL-2 in response to CD3/CD28 when compared to P/I, even though mRNA induction is quite similar. We do not know the reason for this phenomenon, but we want to emphasize that this does not affect our basic findings. We have moved IL-2 induction in Jurkat T cells after CD3/CD28 stimulation to the supplement (Suppl. Fig. 3A and B), because the effect of PPP2R1A knock-down on anti-CD3/CD28 induced IL-2 production is also shown in primary T cells (Figure 2D-F).

Editorial Decision

Thank you for submitting your manuscript to the EMBO Journal. This submission is an invited resubmission of MS 75057 that was rejected post-review earlier this year. Your manuscript has now been seen by the original three referees and their comments are provided below (listed in the same order as on the previous submission). All three referees find that the manuscript has improved, however referee #1 still finds that the data provided is not sufficient to demonstrate the relevance of the observed PP2A mediated regulation of Carma1. Referee #1 suggests identifying a PP2R1A mutant that cannot interact with Carma1. Such a mutant could provide useful insight into the significance of the PP2A/Carma1 interaction. As you have identified, by Y2H analysis, the Carma1 interacting domain in PP2R1a this experiment should not be too difficult to carry out. I find the suggested experiment by referee #1 reasonable and the inclusion of such additional data would strengthen the paper. I would therefore like to ask to address the remaining concern raised by referee #1 in revised version.

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

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

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This is a revised manuscript in which the authors have addressed most of my criticisms in regard to the poor quality and/or inconsistency of the results shown in the paper. However, the loss of function experiments to prove the role of PP2A in the proposed mechanism still are questionable due to the pleitropic actions of this phosphatase in multiple pathways, including its reported actions at different steps of the NF-kB signaling cascades. The fact that some of the previously published data on this topic are controversial (as pointed out by the authors) further reinforce the notion that the use of strategies similar to that carried out in this manuscript can lead to confounding results.

A more definitive proof of these authors' model would have been to identify a PP2R1A mutant that would not interact with Carma and that will act in a dominant negative manner in the pathway. In other words, if by expressing this mutant they would find the same response than in the pharmacological inhibition of PP2A or in the knockdown experiments, then one would be more confident that the biochemical interaction identified here would actually be relevant. Without that solid evidence, the data are interesting but unfortunately not totally definitive.

Referee #2 (Remarks to the Author):
Since authors have addressed all of my concerns/critiques in the previous review, I support to publish the current version of this manuscript in EMBO Journal.

Referee #3 (Remarks to the Author):

The authors have satisfyingly addressed most of my concerns and considerably increased the quality of the experimental data. In my eyes the paper is now acceptable for publication.

Revision - authors' response

16 November 2010

Response to Referee#1

A more definitive proof of these authors’ model would have been to identify a PP2R1A mutant that would not interact with Carma and that will act in a dominant negative manner in the pathway. In other words, if by expressing this mutant they would find the same response than in the pharmacological inhibition of PP2A or in the knockdown experiments, then one would be more confident that the biochemical interaction identified here would actually be relevant. Without that solid evidence, the data are interesting but unfortunately not totally definitive.

We agree that the suggested experiment involving a Carma1 specific dominant-negative mutant of PPP2R1A would indeed present a definitive prove for the proposed model. However, it will be extremely difficult to obtain a PPP2R1A mutant that is not binding to Carma1, but still assembled into a functional PP2A holo-complex. Crystal structures of the PP2A holoenzyme reveal that its assembly and functionality requires large parts of PPP2R1A (Cho et al., Nature 2007, 445:53-57; Xu et al., Cell 2006, 127: 1239-1251). PPP2R1A (589 aa) consists of 15 HEAT repeats (each ~ 39 aa). HEAT repeat 1-10 (aa 8-399) is required for binding the regulatory B subunits and HEAT 11-15 (aa 400-589) interacts with the catalytic C subunit. Binding to other interaction partners and substrates takes place in different regions. Also, it has been shown that mutations that inactive the PP2A holoenzyme are scattered throughout the entire reading frame (e.g. see Sablina et al. Cell 2007, 129:969-982). In the Y2H screen we have identified a PPP2R1A fragment comprising aa 159-589 that is interacting with Carma1. Extensive mutagenesis would be required to map the exact Carma1 interaction surface on PPP2R1A and to obtain an otherwise intact PPP2R1A mutant. It is even unclear, if we would be able to obtain such a mutant.

However, we took up the reviewers suggestions and pursued a different dominant-negative approach using the PPP2R1A deletion fragment 159-589 (PPP2R1A DN) that efficiently binds to Carma1 (Fig. 1B and C and Fig. S1), but is unable to associate with regulatory B subunits and therefore compromised in recruiting a PP2A holo-enzyme complex (Cho et al., Nature 2007, 445:53-57; Xu et al., Cell 2006, 127: 1239-1251). Indeed, PPP2R1A DN is acting in a dominant-negative manner and enhances stimulus dependent NF-kB activation and Carma1 S645 phosphorylation upon overexpression in Jurkat T cells (new Fig. 7A and B). To prove that augmented phosphorylation at S645 in Carma1 is critical for increased NF-kB activation, we again transfected Carma1 deficient JPM50.6 T cells by Carma1 wt, the phospho-mutant S645A or the phospho-mimetic S645E (Fig. 7C). Whereas Carma1 wt mediated NF-kB activation is increased by co-expression of dominant-negative PPP2R1A DN, mutation of the phospho-acceptor sites in S645A and S645E mutants strongly diminishes this increase. Most importantly, the phospho-mimetic Carma1 S645E mutation can restore NF-kB activation, but due to the glutamate substitution this mutant is not prone to dephosphorylation events. Since NF-kB activation of Carma1 S645E is not augmented by PPP2R1A DN, we present strong evidence that dephosphorylation of S645 is a critical event for the negative regulatory action of PP2A. NF-kB activity is not in general enhanced in Carma1 S645E expressing cells, which indicates that a glutamate at this position may not fully compensate for a phosphorylated serine residue at this position. Also the Carma1 S645E mutant still requires P/I stimulation, revealing that phosphorylation at S645 is necessary but not sufficient to trigger NF-kB activation. We have introduced a new paragraph into the Results (page 14-15) and slightly modified the Discussion (page 16 1st paragraph, page 17 2nd paragraph and page 18 1st paragraph) accordingly.
We thank the referee for the critical comment that helped us to design an experiment that lends strong support to our model.

Acceptance Letter 18 November 2010

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #1 to review the revised version and I have now received the comments back. As you can see below, the referee appreciates the added experiment and supports publication here. I am therefore pleased to proceed with the acceptance of the paper for publication here. You will receive the formal acceptance letter shortly.

Sincerely

Editor
The EMBO Journal

REFEREE REPORT
Referee #1

The new experiments using a deltaN mutant of PP2A reinforce the authors' model and make this a more solid paper.