Dephosphorylation of Carma1 by PP2A negatively regulates T-cell activation

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The Carma1–Bcl10–Malt1 (CBM) complex bridges T-cell receptor (TCR) signalling to the canonical I κ B kinase (IKK)/NF- κ B pathway. NF- κ B activation is triggered by PKC θ -dependent phosphorylation of Carma1 after TCR/CD28 co-stimulation. PKC θ -phosphorylated Carma1 was suggested to function as a molecular scaffold that recruits preassembled Bcl10–Malt1 complexes to the membrane. We have identified the serine–threonine protein phosphatase PP2A regulatory subunit α (PPP2R1A) as a novel interaction partner of Carma1. PPP2R1A is associated with Carma1 in resting as well as activated T cells in the context of the active CBM complex. By siRNA-mediated knockdown and *in vitro* dephosphorylation, we demonstrate that PP2A removes PKC θ -dependent phosphorylation of Ser645 in Carma1, and show that maintenance of this phosphorylation is correlated with increased T-cell activation. As a result of PP2A inactivation, we find that enhanced Carma1 S645 phosphorylation augments CBM complex formation, NF- κ B activation and IL-2 or IFN- γ production after stimulation of Jurkat T cells or murine Th1 cells. Thus, our data define PP2A-mediated dephosphorylation of Carma1 as a critical step to limit T-cell activation and effector cytokine production.

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Introduction

The formation of the Carma1–Bcl10–Malt1 (CBM) complex is an essential step in T-cell receptor (TCR)-induced I κ B kinase

(IKK)/NF- κ B activation. TCR/CD28 co-ligation induces the formation of the immunological synapse and activates PKC θ (Monks *et al*, 1997). In turn, Carma1, also known as CARD11, is phosphorylated by PKC θ , which promotes the recruitment of preassembled Bcl10–Malt1 complexes (Matsumoto *et al*, 2005; Sommer *et al*, 2005). Within the CBM complex, Bcl10 and Malt1 are modified by poly-ubiquitination leading to the recruitment and activation of the IKK complex and initiation of canonical NF- κ B signalling (Oeckinghaus *et al*, 2007; Wu and Ashwell, 2008). Activity of the CBM complex is tightly regulated by its association with other proteins, such as TRAF6, Caspase8, PDK1, CSN5, HPK1 and A20, which control phosphorylation and ubiquitination of CBM components as well as CBM–IKK recruitment (Sun *et al*, 2004; Coornaert *et al*, 2008; McCully and Pomerantz, 2008; Brenner *et al*, 2009; Duwel *et al*, 2009; Park *et al*, 2009; Welteke *et al*, 2009).

Carma1 functions as a molecular scaffold that recruits signalling mediators to the membrane in response to T-cell activation. Carma1 contains a C-terminal membrane-associated guanylate kinase (MAGUK) region, which is composed of a PDZ, SH3 and GUK domain (Bertin *et al*, 2001). Even though no direct membrane-binding motif has been identified, the MAGUK region is involved in the membrane linkage of Carma1, by associating with a yet unknown factor. Membrane binding is critical for Carma1 function, because loss of membrane association by a point mutation within the SH3 domain completely abolishes NF- κ B activation in T cells (Wang *et al*, 2004). In the N-terminus, Carma1 contains a caspase recruitment domain (CARD) that mediates the interaction with Bcl10 (Gaide *et al*, 2001). Loss or gain-of-function mutations have been described for an adjacent coiled-coil (CC) domain (Tanner *et al*, 2007; Lenz *et al*, 2008). The central linker region (LR) between the CC and the MAGUK contains the critical phospho-acceptor sites for the activation of Carma1 by PKCs (Matsumoto *et al*, 2005; Sommer *et al*, 2005). *In vitro* analyses have further suggested that PKC phosphorylation of the LR leads to a conformational change that renders the CARD of Carma1 accessible for interaction with the CARD of Bcl10 (Sommer *et al*, 2005). Besides PKC θ , other protein kinases, such as IKK β , HPK1, CamKII or AKT, regulate Carma1 function and may either induce or interfere with T-cell activation (Ishiguro *et al*, 2006; Narayan *et al*, 2006; Shinohara *et al*, 2007; Brenner *et al*, 2009; Moreno-Garcia *et al*, 2009). Taken together, these data indicate that a balance between activating and inhibiting phosphorylation events on Carma1 decides on the activation of downstream signalling pathways. However, a direct link between the cellular Carma1 phosphorylation level and the extent of CBM complex assembly has not yet been shown.

Protein phosphatase 2A (PP2A) belongs to the family of serine–threonine phosphatases that regulates a large variety of cellular processes. The PP2A heterotrimeric holo-enzyme complexes consist of a dimeric core enzyme that comprises a 36-kDa catalytic C subunit (encoded by the genes $C\alpha$ /PPP2CA and $C\beta$ /PPP2CB) and a 65-kDa constant regulatory A subunit

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(encoded by the genes *PR65 α* /*PPP2R1A* and *PR65 β* /*PPP2R1B*). The dimeric PP2A core structure can associate with different regulatory B subunits that are thought to confer substrate specificity (Janssens and Goris, 2001).

PP2A activity is involved in the control of many signalling pathways and it is supposed to function as a tumour suppressor (Wang *et al*, 1998; Calin *et al*, 2000). With respect to NF- κ B signalling, initial *in vitro* experiments using the PP2A inhibitor okadaic acid (OA) indicated a negative regulatory influence on IKK activation (DiDonato *et al*, 1997). However, PP2A can also remove the inhibitory phosphorylation of Ser68 in the IKK regulatory subunit NEMO/IKK γ (Palkowitsch *et al*, 2008) and deletion of a PP2A-binding site on NEMO attenuates IKK activity (Kray *et al*, 2005), indicating that PP2A can also activate the IKK complex. Thus, in cells PP2A seems to mediate opposing effects on IKK activity that either promote or interfere with cytokine-induced NF- κ B activation (Kray *et al*, 2005; Witt *et al*, 2009). Furthermore, by dephosphorylating TRAF2, PP2A can modulate TNF α signalling upstream of the IKK complex (Li *et al*, 2006). In T lymphocytes, pharmacologic inhibition using OA or siRNA-mediated downregulation of the PP2A catalytic subunit C β /PPP2CB resulted in increased cytokine production and NF- κ B activation after T-cell stimulation (Chuang *et al*, 2000; Sun *et al*, 2010). However, a direct functional target of PP2A in TCR/CD28-induced NF- κ B signalling has not yet been identified.

Here, we report that the PP2A regulatory subunit A (PPP2R1A) directly interacts with the CBM complex in activated T cells. PP2A counteracts the activating phosphorylation on Ser645 of Carma1. Congruently, downregulation of PP2A enhances CBM complex assembly post-induction and thereby augments T-cell activation. These results define PP2A as a negative regulator of upstream NF- κ B signalling in T cells and provide evidence that assembly and activity of the CBM complex in cells is causally linked to the level of Carma1 phosphorylation.

Results

PP2A constant regulatory subunit A associates with Carma1

To search for novel regulators of Carma1, we performed yeast-two-hybrid (Y2H) screens using either the C-terminal GUK domain (aa 932–1147) or the entire C-terminus (aa 600–1147) containing PDZ, SH3 and GUK domains of Carma1 as bait. In both screens we identified a fragment of the PP2A regulatory subunit A PPP2R1A (aa 159–589) as a new interaction partner for Carma1 (Supplementary Figure S1). PPP2R1A is composed of 15 α -helical 38 aa long HEAT repeats that serve as an extended interaction surface and the identified fragment lacks the N-terminal HEAT repeat 1–4 (aa 8–161).

To investigate whether Carma1 and PPP2R1A associate in cells, we performed co-immunoprecipitation (co-IP) experiments after transfection of HEK293 cells. Indeed Flag-PPP2R1A interacted with full-length HA-Carma1 after either anti-HA or anti-Flag IP (Figure 1A–C). Carma1 interacted with full-length PPP2R1A as well as with the fragment 159–589 identified by Y2H. Congruent with the results from the Y2H screen, PPP2R1A binds to the C-terminus, but not to the N-terminus of Carma1. Strongest interaction was observed with a fragment comprising the C-terminal GUK

domain (aa 932–1147) and deletion of the GUK domain (construct aa 1–931) prevented binding of Carma1 to PPP2R1A (Figure 1B). Fine mapping showed that PPP2R1A associates with the very C-terminal part of the Carma1 GUK domain (aa 1031–1147) (Supplementary Figure S2; Figure 1D).

PP2A acts as a negative regulator of IL-2 induction and NF- κ B signalling in T cells

To address a potential role for PPP2R1A–Carma1 interaction, we first evaluated the involvement of PP2A in NF- κ B signalling and T-cell activation. Previous data have suggested a negative regulatory function of PP2A for T-cell activation, but these studies largely relied on the usage of the pharmacologic PP2A inhibitor OA, which was shown to potently inhibit other phosphatases besides PP2A (Zolnierowicz, 2000; Shui *et al*, 2007). Since the catalytic activity of PP2A critically depends on the association between the constant regulatory A subunit PPP2R1A and the catalytic subunit PPP2CA (Hombauer *et al*, 2007), we took an siRNA approach to knockdown both subunits in Jurkat T cells. The constant regulatory and catalytic PP2A subunits are encoded by two distinct isoforms (α or A and β or B) that share high-sequence identity on the protein level. The two α isoforms are the major isoforms expressed in adult cells and tissues (Khew-Goodall and Hemmings, 1988; Hemmings *et al*, 1990). We generated two siRNAs that specifically target PPP2R1A and one siRNA to knockdown PPP2CA that all caused downregulation of their respective targets (Figure 2A). Since the anti-PPP2R1A and anti-PPP2CA antibodies cross-react with A and B isoforms, we demonstrated that the siRNAs promoted efficient downregulation of the overall PPP2R1A/B and PPP2CA/B amounts (Figure 2A). The remaining protein may be derived from either the residual A isoforms or the more weakly expressed B isoforms. Further, downregulation of one PP2A subunit also induced a reduction in the amounts of the other subunit, indicating a destabilization of the PP2A holo-enzyme complex.

To determine the effect of PP2A knockdown on T-cell activation, we measured the accumulation of IL-2 protein by ELISA in the supernatant of cells stimulated with PMA/Ionomycin (P/I) or by CD3/CD28 co-ligation (Figure 2B; Supplementary Figure S3A). Compared with siGFP control transfected cells, knockdown of PPP2R1A or PPP2CA augmented expression of secreted IL-2 after P/I and CD3/CD28 stimulation. We used quantitative RT-PCR to determine, if the increased IL-2 expression is caused by an enhanced upregulation of the *IL-2* mRNA (Figure 2C; Supplementary Figure S3B). Indeed, knockdown of PPP2R1A caused an increase in *IL-2* mRNA induction upon P/I or CD3/CD28 stimulation. To confirm the effects of PP2A in primary T cells, we adenovirally transduced murine CD4⁺ T cells from DO11.10 TCR-transgenic mice that also express a signalling-inactive form of the human coxsackie adenovirus receptor as a second transgene. T cells from these mice are able to take up recombinant adenoviruses (Wan *et al*, 2000) and ectopically express adenovirus encoded transgenes or small-hairpin RNAs in a transient manner. This system was shown to permit efficient knockdown specifically in cells that have high levels of a vector co-expressed GFP marker (Glasmacher *et al*, 2010), indicating that increased shRNA expression may be able to overcome the observed lower knockdown efficiency in peripheral mouse CD4 T cells (Oberdoerffer *et al*, 2005). Purified CD4⁺ T cells were infected with

Figure 1 PPP2R1A interacts with Carma1 in overexpression experiments. (A) Interaction of overexpressed Carma1 aa 932–1147 (GUK), Carma1 aa 600–1147 (C-term), Carma1 full length with PPP2R1A full length. HEK 293 cells were co-transfected with Flag-PPP2R1A and HA-Carma1 constructs. Immunoprecipitation (IP) was carried out using HA-beads and analysed by western blotting. Strongest interaction is detectable between PPP2R1A and C-term/GUK domain of Carma1. (B) GUK region of Carma1 is important for PPP2R1A binding. HEK293 cells were co-transfected with Flag-PPP2R1A (aa 159–589) and different HA-tagged Carma1 constructs as indicated (compare also (D)). Binding of PPP2R1A to Carma1 fragments was analysed by western blotting after HA IP. The C-terminal GUK domain of Carma1 is required for efficient interaction with PPP2R1A. (C) 293 cells were co-transfected with Flag-PPP2R1A (aa 159–589) and different HA-tagged Carma1 constructs as indicated. Binding of PPP2R1A to Carma1 fragments was analysed by western blotting after anti-Flag IP. The asterisk indicates migration of IgGs. Strongest interaction is detectable with Carma1 aa 932–1147 (GUK), no interaction with Carma1 aa 1–623 (N-term). (D) Schematic presentation of the interaction between Carma1 fragments and PPP2R1A (ND, not determined).

adenoviruses that encode control or PPP2R1A-specific shRNAs, prior to stimulation with plate bound anti-CD3/CD28 antibodies under Th1 conditions. Downregulation of PPP2R1A by the shRNA was verified by western blotting after FACS sorting of the 10% highest GFP-expressing T cells (Figure 2D). By intracellular staining and single cells FACS analysis we determined the frequency of IL-2- and IFN γ -producing T cells during anti-CD3/CD28 re-stimulation performed 5 or 6 days after isolation and infection (Figure 2E and F). When compared with control cells, knockdown of PPP2R1A resulted in a higher number of IL-2 or IFN γ expressing as well as IL-2/IFN γ co-expressing cells as well as in an overall increase in mean fluorescence that was observed by FACS staining with antibodies against IFN γ and to a lesser extent with antibodies against IL-2. Our data consistently show that the frequency of IL-2 and IFN γ -positive cells increase between 1.5- and 3-fold due to expression of an shRNA against PPP2R1A, with stronger effects being visible in re-stimulations and cytokine measurements on day 6 after T-cell isolation and within the population of double-positive IL-2/IFN γ -producing cells (Figure 2F). Our results provided evidence that the phosphatase PP2A acts as a negative regulator of Th1 effector cytokine production in response to TCR/CD28 co-stimulation.

Since we had identified PPP2R1A as an interactor of the essential NF- κ B signalling mediator Carma1 in T cells, we analysed the effects of PP2A knockdown on NF- κ B activation. siRNA-mediated depletion of PPP2R1A or PPP2CA promoted stronger NF- κ B DNA-binding activity in Jurkat T cells after stimulation with CD3/CD28 (Figure 2G; Supplementary Figure S4A). NF- κ B DNA-binding activity after T-cell activation largely consisted of p50/p65 heterodimers as determined by supershift analysis (Supplementary Figure S4B). To determine whether enhanced upstream signalling is responsible for augmented NF- κ B DNA binding, we determined the extent of IKK α / β phosphorylation after PPP2R1A downregulation. Indeed, activating T-loop phosphorylation in response to P/I stimulation was enhanced in PPP2R1A knockdown cells (Figure 2H). Thus, PP2A acts as a negative regulator of upstream NF- κ B signalling and subsequent IL-2 production.

IL-2 induction also relies on calcium-dependent activation of NF-AT in T cells. To see if PP2A also affect NF-AT signalling pathway, we measured the cytosolic calcium release after stimulation with CD3/CD28 in PPP2R1A knockdown cells (Supplementary Figure S5A). siPPP2R1A knockdown T cells compared with siGFP cells showed no increase of the calcium influx from intracellular stores or over the plasma

Figure 2 PP2A regulates IL-2 production and NF- κ B activity in Jurkat and in primary T cells. (A) Jurkat T cells were transfected with siRNA against GFP (control), two independent siRNAs against PPP2R1A (1 and 2) or against PPP2CA. Protein expressions after knockdown are depicted. Note that PPP2R1A or PPP2CA knockdown also reduces protein levels of other subunit. (B) Jurkat T cells were transfected with siRNA against GFP control, PPP2R1A or PPP2CA and stimulated with P/I. Secreted IL-2 amounts were measured by ELISA. Downregulation of PPP2R1A enhances IL-2 production. (C) Jurkat T cells were transfected with siRNAs against GFP or PPP2R1A and stimulated with P/I for 3 h. RNA was isolated and IL-2 transcript levels were investigated by quantitative RT-PCR. IL-2 mRNA level in siPPP2R1A-transfected cells is increased. Transcript levels were normalized using RNA polII mRNA. (D) Primary CD4⁺ T cells were transduced with adenoviruses encoding for GFP and shRNA against PPP2R1A-1 or control. T cells with high co-expression of GFP (top 10%) were sorted by FACS and PPP2R1A expression after knockdown was determined by western blotting. (E) CD4⁺ T cells were adenovirally transduced as indicated and stimulated with plate bound anti-CD3/CD28 for 6 h. Intracellular IFN γ and IL-2 in GFP-positive cells (top 10%) was determined by FACS. (F) Comparison of the relative numbers of IL-2 and IFN γ -positive CD4⁺ T cells after PPP2R1A knockdown in the different gates (IL-2-positive, IFN γ -positive or IL-2/IFN γ double-positive cells). PPP2R1A-1 knockdown resulted in higher number of IFN γ and IL-2-producing cells. (G) Jurkat T cells were transfected with siRNA against GFP, PPP2R1A-2 or PPP2CA and stimulated with anti-CD3/CD28 antibodies as indicated. NF- κ B DNA binding was determined by EMSA. PP2A knockdown enhances NF- κ B activation. (H) Jurkat T cells were transfected with siRNA against GFP or PPP2R1A-2 and stimulated with P/I as indicated. IKK T-loop phosphorylation was detected after anti-IKK α IP. PPP2R1A knockdown augments IKK activation.

membrane after stimulation. An increase in intracellular calcium is inducing activation of NF-AT, which is mediated by the phosphatase calcineurin. We find no significant changes in nuclear NF-AT DNA binding in PPP2R1A or PPP2CA knockdown cells (Supplementary Figure S5B), indicating that PP2A is predominately balancing NF- κ B signaling to inhibit IL-2 induction.

PPP2R1A/B constitutively associates with Carma1 in T cells

To determine whether PPP2R1A association to Carma1 is involved in the negative regulatory function of PP2A, we first investigated association of endogenous PPP2R1A/B to Carma1. For this we performed co-IPs from Jurkat and Carma1-deficient JPM50.6 T cells (Figure 3A). Carma1

Figure 3 PPP2R1A/B interacts with endogenous Carma1 before and after T-cell stimulation. **(A)** Interaction of Carma1 and PPP2R1A/B in Jurkat T cells. Jurkat T cell or Carma1-deficient JPM50.6 cells were untreated or P/I treated as indicated and after lysis subjected to anti-Carma1 IP. PPP2R1A/B binds to Carma1 independent of stimulation. **(B)** Jurkat T cell or Carma1-deficient JPM50.6 cells were treated as in **(A)** prior to PPP2R1A/B or IgG control IP. Carma1 co-precipitates with PPP2R1A independent of stimulation, but associated Carma1 is hyper-phosphorylated after 15 min of P/I stimulation. **(C)** PPP2R1A/B is recruited to the high molecular weight CBM complex. Fractions of unstimulated or P/I-stimulated Jurkat T cells were separated by gel filtration (see Supplementary Figure S6). Pools containing the CBM complex (pool I), Carma1 (pool II) or Bcl10–Malt1 (II) were subjected to anti-Bcl10 IP to monitor binding of PPP2R1A. High amounts of PPP2R1A/B are associated with the active CBM complex (pool I) only after stimulation. **(D)** PPP2R1A/B–CBM interaction requires Carma1. Jurkat T cells or JPM50.6 cells were stimulated with P/I, lysed as above and subjected to Bcl10 IP. **(E)** Anti-CD3/CD28 ligation induces PPP2R1A/B–CBM interaction in Jurkat T cells. Jurkat T cells were stimulated by anti-CD3/CD28 ligation. Cells were lysed in co-IP buffer followed by anti-Bcl10 or IgG control IP. After T-cell activation, PPP2R1A is recruited to the CBM complex. **(F)** PPP2R1A/B–CBM interaction in primary mouse T cells. Peripheral T cells were stimulated as indicated. IP was performed as described above. PPP2R1A/B association to the CBM complex increases after stimulation.

associated with PPP2R1A in unstimulated and stimulated Jurkat T cell. Congruently, Carma1 was co-precipitated after PPP2R1A IP from extracts of Jurkat T cells also independent of stimulation (Figure 3B). Interestingly, PPP2R1A-associated Carma1 appears to be transiently hyper-phosphorylated, as indicated by a slight retardation in the SDS gel after 15 min of P/I stimulation (see Figure 4A).

Since formation of the high molecular weight Carma1–Bcl10–Malt1 (CBM) complex is essential for NF- κ B activation in T cells, we wanted to determine whether PPP2R1A indeed interacts with the CBM holo-complex. We used Superose6 size exclusion chromatography to separate cellular protein complexes from extracts of untreated or P/I-stimulated Jurkat T cells (Oeckinghaus *et al*, 2007). Eluted fractions were analysed by western blotting for the presence of Carma1, Malt1, Bcl10 and PPP2R1A/B (Supplementary Figure S6). We pooled fraction 11–14 containing the stimulus-dependent CBM holo-complex (I), fractions 20–23 containing Carma1 (II) and fractions 24–27 containing Bcl10–Malt1 (III). To enrich CBM complex components, we immunoprecipitated the pooled fractions with an anti-Bcl10 antibody that was shown to efficiently precipitate the CBM complex (Wegener *et al*, 2006; Figure 3C). PPP2R1A/B was not co-precipitated with Bcl10 in the pool III containing the low molecular weight Bcl10–Malt1 complexes (fractions 24–27).

Also in the pool II containing Carma1 that is not bound to Bcl10 (fractions 20–23), PPP2R1A/B was not detected after Bcl10 IP. However, PPP2R1A/B and Carma1 co-precipitated with Bcl10 in the high molecular weight pool I (fractions 11–14) only after P/I stimulation, demonstrating a strong interaction of PPP2R1A/B with the CBM holo-complex in activated T cells. To see whether Carma1 is necessary for the interaction with the CBM holo-complex, we compared stimulus-dependent association of PPP2R1A with Bcl10 in Jurkat T cells and Carma1-deficient JPM50.6 T cells (Figure 3D). Again, PPP2R1A–Bcl10 association strictly required the presence of Carma1, indicating the crucial role of Carma1 for a stimulus-dependent recruitment of PPP2R1A/B to the CBM complex. We also detected stimulus-dependent association of PPP2R1A to Bcl10 in the context of the CBM in response to CD3/CD28 co-stimulation (Figure 3E) as well as in primary murine CD4⁺ T cells after P/I stimulation (Figure 3F). These data suggest that PPP2R1A/B interacts directly with Carma1 in resting as well as in activated T cells in the context of the CBM complex.

PP2A mediates dephosphorylation of Carma1

Previous studies showed phosphorylation of Carma1 on several serine residues in the LR upon antigenic stimulation of B and T cells (Matsumoto *et al*, 2005; Shinohara *et al*,

Figure 4 PPP2R1A negatively regulates Carma1 phosphorylation. (A) Carma1 phosphorylation visualized by λ phosphatase treatment. Jurkat T cells were stimulated with P/I, lysed in co-IP buffer and anti-Bcl10 IP was performed. After washing, IP beads were incubated with λ phosphatase and migration of Carma1 was analysed by WB. λ Phosphatase induces a faster migration of Bcl10-associated Carma1, which indicates hyper-phosphorylation of Carma1 (H.i., heat inactivated). (B) Carma1 is phosphorylated on Ser645 after T-cell activation. Jurkat T cells were stimulated with CD3/CD28 as indicated. After Carma1 IP, Carma1 phosphorylation was detected by phospho-Ser645-specific antibody. (C) Ser645 and the GUK domain are required for NF- κ B activation. Carma1-deficient JPM50.6 cells were transfected with empty vector, Carma1 wt, Carma1 S645A, Carma1 Δ GUK (1–931) or Carma1 Δ 100 (1–1048) and NF- κ B luciferase reporter gene. Prior to lysis, the cells were stimulated with P/I (4 h) and luciferase activity was measured. C-terminal deletions and mutation of Ser645 prevents NF- κ B activation. Shown is the average of two independent experiments and expression control by western blot. (D, E) PPP2R1A and PPP2CA impair phosphorylation of Carma1 on Ser645. Jurkat T cells were transfected with GFP, PPP2R1A (D) or PPP2CA (E) targeting siRNAs and stimulated with CD3/CD28 as indicated. Lysis and detection of phospho-Carma1 was performed as in (B). Stimulus-dependent Carma1 Ser645 phosphorylation is enhanced after PP2A downregulation. (F) PP2A inhibitor okadaic acid (OA) increases Carma1 Ser645 phosphorylation. Jurkat T cells were incubated for 30 min with 250 nM OA prior to anti-CD3/CD28 stimulation and lysis. Ser645 phosphorylation in Carma1 was analysed after Carma1 IP and PKC θ Thr538 phosphorylation in the lysates. (G) PPP2R1A knockdown does not affect PKC θ Thr538 phosphorylation upon CD3/CD28 co-ligation. Phosphorylation of PKC θ Thr538 was analysed by western blotting after control or PPP2R1A knockdown in Jurkat T cells as indicated. (H) Recruitment of PKC θ to Carma1 in activated T cells. Extracts from Jurkat T cells after P/I stimulation were subjected to either PKC θ (left) or Carma1 (right) IP before detection of co-precipitated proteins. (I) Transient association of Carma1–PKC θ after T-cell activation. P/I stimulation for indicated times, lysis and Carma1 co-IP were performed as in (H).

2007). Although negative regulatory phosphorylations of Carma1 have been observed as well (Moreno-Garcia *et al*, 2009), most phosphorylation events have activating effects and were suggested to cause a conformational change, which allows the recruitment of the Bcl10–Malt1 to Carma1. Indeed, comparison of Carma1 from Jurkat T-cell lysates and after anti-Bcl10 IP revealed that Bcl10–Malt1-associated Carma1 is migrating more slowly in an SDS–PAGE (Figure 4A). Further, the retarded Carma1 band can be converted to a faster migration by λ phosphatase treatment, demonstrating that Carma1 within the CBM complex is hyper-phosphorylated.

To analyse the influence of PP2A on the phosphorylation status of Carma1, we used a Carma1 phosphorylation-specific antibody that specifically recognizes phospho-Ser645 of human Carma1 (Shinohara *et al*, 2007). Phosphorylation of Ser645 (corresponding to chicken Ser668 and murine Ser657) within the LR of Carma1 is catalysed by PKC θ in T cells and PKC β in B cells (Sommer *et al*, 2005; Shinohara *et al*, 2007). To visualize Carma1 phosphorylation, we performed Carma1 IPs from Jurkat T-cell extracts prepared after CD3/CD28 stimulation followed by anti-phospho-Ser645 western blotting. Carma1 Ser645 phosphorylation was not detectable

Figure 5 PPP2CA dephosphorylates Ser645 in Carma1. **(A)** Carma1 IP was performed after anti-CD3/CD28 stimulation of Jurkat T cells. Precipitates were incubated with recombinant PPP2CA before Ser645 phosphorylation of Carma1 was analysed by western blot. **(B)** Catalytic activity of PPP2CA is required for Carma1 dephosphorylation. Jurkat T cells were transfected with HA-PPP2CA wt or HA-PPP2CA mut (H118N; L199P). After anti-CD3/CD28 stimulation parallel anti-Carma1 and anti-HA IP was performed. Dephosphorylation reaction was carried out in the precipitates before detection of P-Ser645 in Carma1.

in unstimulated Jurkat T cells, but became visible and peaked between 5 and 15 min after T cells co-stimulation (Figure 4B). By reconstitution of the Carma1-deficient Jurkat T cell line JPM50.6 (Wang *et al*, 2002), we examined the requirement of the Ser645 of human Carma1 for NF- κ B signalling in T cells (Figure 4C). Alanine substitution of serine 645 completely abolished the ability of Carma1 to rescue NF- κ B activation in response to P/I stimulation. Interestingly, also deletion of the GUK domain or the very C-terminal 100aa of Carma1 that comprise the PPP2R1A-binding surface (compare Figure 1) led to a loss of function (Figure 4C), indicating that the Carma1 C-terminus is not just simply recruiting a negative regulator, but is itself essential for mediating NF- κ B activation.

We asked if PP2A is regulating Ser645 phosphorylation of Carma1 by downregulation of PPP2R1A or PPP2CA in Jurkat T cells using siRNAs (Figure 4D and E). Indeed, reduced PPP2R1A or PPP2CA amounts caused enhanced Carma1 phosphorylation of Ser645 after CD3/CD28 stimulation (Figure 4D and E). To prove that catalytic activity of PP2A is required for counteracting Carma1 Ser645 phosphorylation, we incubated the Jurkat T cells with the PP2A inhibitor OA prior to stimulation (Figure 4F). Also, PP2A inhibition promoted an enhanced Carma1 phosphorylation after CD3/CD28 co-ligation, providing evidence that PP2A controls the level and the duration of Carma1-activating phosphorylation.

Since PKC θ acts directly upstream of Carma1 and catalyses phosphorylation of Ser645 after T-cell activation (Matsumoto *et al*, 2005; Sommer *et al*, 2005), we wanted to determine the influence of PPP2R1A knockdown on PKC θ activation. We monitored phosphorylation of Thr538 in the activation loop of PKC θ . Thr538 phosphorylation is critical for PKC θ activity and NF- κ B activation in response to CD3/CD28 co-engagement (Liu *et al*, 2002). Neither the level nor the kinetic of PKC θ Thr538 phosphorylation was significantly altered after OA treatment or after PPP2R1A knockdown (Figure 4F and G). Further, CD3/CD28-induced activation of PKC θ was not significantly enhanced in PPP2R1A knockdown cells as monitored by an *in vitro* kinase assay after PKC θ IP using myelin basic protein as a substrate (Supplementary Figure S7). These data indicate that PP2A is not reducing upstream signalling,

but directly modulating Carma1 phosphorylation in activated T cells.

The constitutive association between PPP2R1A/B and Carma1 raises the question how the balance between phosphorylation and dephosphorylation of Carma1 is regulated upon T-cell stimulation. We therefore determined binding of the activator kinase PKC θ to Carma1 by co-IP. PKC θ was recruited to Carma1 after P/I stimulation (Figure 4H). PKC θ -Carma1 association was transient and correlated with the peak of Carma1 Ser645 phosphorylation (Figure 4I; compare Figure 4B), suggesting that Carma1 phosphorylation is initiated by the induced proximity of the activating kinase.

To address if PP2A can act as a direct Carma1 phosphatase, we performed *in vitro* dephosphorylation assays using recombinant PPP2CA. After anti-Carma1 IP from Jurkat T cells, we incubated increasing concentrations of recombinant PPP2CA to the precipitates prior to the detection of Ser645-phosphorylated Carma1 (Figure 5A). Recombinant PPP2CA was able to dephosphorylate Ser645 in Carma1 *in vitro*. To prove that the catalytic activity of PPP2CA is required for Carma1 dephosphorylation, we transfected HA-PPP2CA wt or the catalytically inactive HA-PPP2CA mut (H118N; L199P) into Jurkat T cells (Figure 5B). After lysis, Carma1 and HA-PPP2CA were immunoprecipitated in parallel with anti-HA and anti-Carma1 antibodies in the presence of phosphatase inhibitors. Phosphatase inhibitors were removed by washing before dephosphorylation reaction on the precipitates. Western blot detection of P-Ser645 Carma1 revealed that PPP2CA wt, but not the catalytic centre mut PPP2CA was able to remove phosphorylation from Ser645 on Carma1, providing evidence that PP2A can act as a direct Carma1 phosphatase.

PP2A impairs CBM complex assembly

Phosphorylation of the Carma1 LR is a key event for the formation of the CBM complex (Matsumoto *et al*, 2005; Sommer *et al*, 2005). Mutation of chicken Ser668 (human Ser645) in Carma1 in DT40 B cells is sufficient to inhibit CBM complex formation, which demonstrates the importance of this phospho-acceptor site (Shinohara *et al*, 2007). We therefore determined the effect of PPP2R1A knockdown on the

Figure 6 PP2A is required for post-inductive destabilization of the CBM complex. Jurkat T cells were transfected with siRNA against GFP or PPP2R1A-1 (**A, B**) or PPP2R1A-2 (**C**) and stimulated with CD3/CD28 (**A**) or P/I (**B, C**) as indicated. After lysis in co-IP buffer and anti-Bcl10 IP, the CBM and PP2A complexes were visualized by western blotting. In cells transfected with siPPP2R1A, stimulus-dependent recruitment of Bcl10 to Carma1 is enhanced. Co-precipitation of PP2A regulatory and catalytic subunits is reduced after PPP2R1A knockdown. (**D**) PP2A inhibitor okadaic acid (OA) augments CBM complex formation. Jurkat T cells were incubated for 30 min with 250 nM OA prior to P/I stimulation and lysis. Bcl10–Carma1 association was analysed as in (**A–C**).

association of the CBM complex in activated Jurkat T cells. Again, CBM complex formation was monitored by Bcl10 IPs in PPP2R1A knockdown cells after T-cell activation with P/I or CD3/CD28 (Figure 6). As noted earlier, PPP2R1A is recruited to Bcl10 and the CBM complex upon T-cell activation. As expected, association of the PP2A regulatory subunits PPP2R1A/B with the CBM is diminished in PPP2R1A knockdown cells. In addition, binding of the catalytic subunit of PP2A (PPP2CA) is detectable after Bcl10 IP and depends on the presence of the regulatory subunit PPP2R1A (Figure 6A and B). Clearly, PPP2R1A downregulation by the two independent siRNAs increased association of Carma1 and Bcl10 after CD3/CD28 or P/I stimulation (Figure 6A–C). Especially in the case of CD3/CD28 co-ligation, PPP2R1A knockdown led to a sustained recruitment of Bcl10 to Carma1 (Figure 6A). In addition, pharmacological inhibition of PP2A by OA promoted an increased binding of Bcl10 to Carma1 (Figure 6D). Taken together, our results demonstrate that PP2A is critical for the removal of Ser645-activating phosphorylation from Carma1 and thereby negatively regulates CBM complex formation in T cells.

PP2A counteracts NF- κ B activation triggered by Carma1 phosphorylation

To prove that PP2A catalysed dephosphorylation of Carma1 and diminished CBM assembly is responsible for its negative regulatory effect on NF- κ B activation, we performed overexpression of the PPP2R1A 159–589 (PPP2R1A Δ N) in Jurkat T cells. This deletion mutant binds Carma1 (see Figure 1B and C), but it lacks the complete N-terminus and is therefore unable to recruit a PP2A holo-enzyme complex (Xu *et al*, 2006; Cho and Xu, 2007). Thus, PPP2R1A Δ N might function

as a dominant-negative mutant that interferes with PP2A catalysed Carma1 dephosphorylation. Congruently, Jurkat T cells transfected with PPP2R1A Δ N showed an enhanced NF- κ B activation after P/I or CD3/CD28 co-stimulation in reporter assays when compared with control cells or PPP2R1A wt expressing cells (Figure 7A). Thus, PPP2R1A Δ N can indeed act as a dominant-negative repressor of PP2A activity in the NF- κ B pathway. Next we tested, if increased NF- κ B activation after transfection of PPP2R1A Δ N coincided with an enhanced Carma1 phosphorylation (Figure 7B). Again, stronger Carma1 phosphorylation on Ser645 was detected in Jurkat T cells overexpressing PPP2R1A Δ N after anti-CD3/CD28 co-ligation. To prove that dephosphorylation S645 in Carma1 is a critical event for the negative regulatory function of PP2A, we again performed NF- κ B reporter assays in Carma1-deficient JPM50.6 cells (Figure 7C). As shown earlier, expression of Carma1 wt but not Carma1 S645A was able to rescue NF- κ B activation in JPM50.6 cells. Further, transfection of a phospho-mimetic Carma1 S645E mutant could restore P/I-induced NF- κ B activation in the Carma1-deficient Jurkat T cells. Again, expression of PPP2R1A Δ N augmented NF- κ B activation in JPM50.6 cells expressing Carma1 wt. However, PPP2R1A Δ N was unable to enhance NF- κ B activity in cell transfected with Carma1 phospho-mutant S645A or the Carma1 phospho-mimetic S645E. Carma1 S645E mutant still requires P/I stimulation to activate NF- κ B, revealing that phosphorylation at S645 is necessary but not sufficient to trigger downstream signalling. As the activity of Carma1 S645E mutant is not augmented by PPP2R1A Δ N, these data provide evidence that PP2A catalysed dephosphorylation of S645 in Carma1 is indeed critical for counteracting NF- κ B signalling upon T-cells activation.

Figure 7 Dominant-negative PPP2R1A Δ N enhances NF- κ B activation by increasing phosphorylation of S645 in Carma1. **(A)** Expression of PPP2R1A Δ N (159–589) enhanced NF- κ B activation upon T-cells stimulation. Jurkat T cells were transfected with empty vector (mock), Flag-PPP2R1A Δ N or Flag-PPP2R1A wt and NF- κ B luciferase reporter gene. Luciferase activity was measured after 4 h of P/I or CD3/CD28 stimulation. Flag-PPP2R1A Δ N but not Flag-PPP2R1A augmented NF- κ B activation. All data represent the mean and s.d. of three independent experiments. **(B)** Carma1 phosphorylation on Ser645 is enhanced by PPP2R1A Δ N. Mock or Flag-PPP2R1A Δ N transfected Jurkat T cells were stimulated with CD3/CD28 as indicated and lysates were subjected to Carma1 IP. Detection with the phospho-Ser645-specific antibody shows increased Carma1 phosphorylation in PPP2R1A-expressing cells. **(C)** PPP2R1A Δ N enhances NF- κ B activation through S645 in Carma1. Carma1-deficient JPM50.6 cells were transfected with empty vector (control), Carma1 wt, Carma1 S645A or Carma1 S645E alone or together with Flag-PPP2R1A Δ N. Luciferase activity was determined as in **(A)**. Increased NF- κ B activation by PPP2R1A Δ N requires S645 in Carma1. All data represent the mean and s.d. of four independent experiments. Significance was evaluated by a two-tailed *t*-test (Mann–Whitney); * denotes significant (0.03), NS, denotes not significant (0.2).

Discussion

In this study we show that the PP2A regulatory subunit PPP2R1A binds to the signalling mediator Carma1 to recruit the catalytic subunit PPP2CA to the CBM complex in activated T cells. PP2A is responsible for the dephosphorylation of the activating phosphorylation on Ser645 in the central LR of Carma1. Phosphorylation of Ser645 in Carma1 is a key event for PKC-driven Carma1 activation in T or B cells in response to antigenic stimulation (Matsumoto *et al*, 2005; Sommer *et al*, 2005; Shinohara *et al*, 2007). Consequently, depletion of the PP2A regulatory or catalytic subunits as well as pharmacological inhibition by OA in T cells enhances stimulus-dependent CBM complex assembly, which augments NF- κ B activation and IL-2 production. Further, expression of a dominant-negative PPP2R1A mutant enhances the ability of Carma1 wt, but not of the phospho-mimetic Carma1 S645E mutant, to activate NF- κ B. Thus, dephosphorylation of Carma1 by PP2A serves a critical negative regulatory function in T-cell activation.

The Carma1 LR was shown to act as a negative regulatory domain by forming an intramolecular link with the CARD-CC domain of Carma1. This interaction was proposed to prevent CARD-CARD binding of Carma1 to Bcl10 in resting T cells. LR phosphorylation of Ser552 and Ser645 in human Carma1 by PKC θ reduced the affinity between LR and CARD-CC *in vitro*, suggesting that the phosphorylation eliminates a negative regulatory function of the LR (Sommer *et al*, 2005). Congruent with this, phosphorylation of Ser668 in chicken Carma1 (corresponds to Ser645 in human Carma1) is critical for the B-cell receptor-induced recruitment of Bcl10 to Carma1 in chicken DT40 cells (Shinohara *et al*, 2007). We find that Ser645 of Carma1 is also critical for NF- κ B activation in T cells and that increased Ser645 phosphorylation of Carma1 in PPP2R1A knockdown cells coincides with an augmented recruitment of Bcl10 to Carma1 after TCR/CD28 co-engagement. By showing that PP2A is a negative regulator

of CBM complex formation, we demonstrate that the strength and the duration of Carma1 LR phosphorylation is indeed controlling cellular association of Carma1 and Bcl10 after T-cell activation. Thus, the identification of PP2A as a Carma1 phosphatase lends *in vivo* support to the proposed model of a phosphorylation-dependent conformational switch of Carma1 that triggers the assembly of the CBM complex.

Different protein kinases have been suggested to activate or inactivate Carma1 by phosphorylating various residues inside or outside the LR (Ishiguro *et al*, 2006; Narayan *et al*, 2006; Shinohara *et al*, 2007; Brenner *et al*, 2009; Moreno-Garcia *et al*, 2009). Biochemical and genetic studies indicate that PKC θ is the major kinase that catalyses TCR/CD28-induced phosphorylation in the Carma1 LR (Sun *et al*, 2000; Matsumoto *et al*, 2005; Sommer *et al*, 2005). PP2A inactivation does not enhance activation of PKC θ after TCR/CD28 co-stimulation. Furthermore, PP2A also impedes CBM signalling after P/I stimulation, which directly activates PKC θ and calcium flux in T cells, and thereby bypasses TCR and CD28 co-receptor proximal signalling events. By its ability to remove Ser645 phosphorylation in Carma1, we suggest that PP2A indeed acts at the level of Carma1 for mediating a negative effect on T-cell activation. The importance of dephosphorylating Ser645 is highlighted by the inability of dominant-negative PPP2R1A Δ N to increase the activity of a S645E phospho-mimetic Carma1 mutant. Nevertheless, to execute its negative regulatory role, PP2A may also act on other phosphorylated sites, for example Ser552 that is also phosphorylated by PKC θ , which could even amplify the effects. Despite the fact that PP2A may either directly or indirectly regulate opposing functions by dephosphorylating different substrate residues, our data also in primary Th1 cells clearly indicate that the dominant function of PP2A is to counteract NF- κ B activation and cytokine production after TCR/CD28 co-engagement.

PPP2R1A associates with the C-terminal part of the GUK domain of Carma1. We find that deletion of the C-terminus

abolishes the ability of Carma1 to mediate NF- κ B activation in response to T-cell stimulation. Thus, the C-terminus is not only serving a negative function, but is also essential for TCR/CD28 signalling. Endogenous Carma1 and PPP2R1A are constitutively associated, indicating that PP2A may also function in preventing Carma1 phosphorylation and CBM complex assembly in resting T cells. The constitutive PP2A–Carma1 association raises the question how PP2A is prevented from dephosphorylating Carma1 after initial T-cell stimulation. Our data suggest that induced activation and Carma1 recruitment of PKC θ shifts the balance towards Carma1 hyper-phosphorylation by a transient increase in kinase activity at the site of action. Once PKC θ has been removed from Carma1, constitutively associated PP2A can revert the activating phosphorylation to prevent unbalanced signalling. By its constitutive association, PP2A may also prevent Carma1 phosphorylation and CBM complex assembly in the absence of antigenic stimulation and may thereby counteract chronic T-cell activation. It is also possible that further regulatory events control PP2A activity. PP2A substrate specificity depends on the association of regulatory B subunits (Eichhorn *et al*, 2009). Since we show that a PPP2R1A mutant (PPP2R1A Δ N), which is unable to interact with a B subunit, enhances Carma1 Ser645 phosphorylation, recruitment of a PP2A holo-enzyme complex is crucial for catalysing Carma1 dephosphorylation. Thus, induced activity of a regulatory B subunit to PPP2R1A may be required to activate PP2A upon T-cells stimulation. Importantly, we show that the constant regulatory PPP2R1A subunit is primarily responsible for the recruitment of the PP2A holo-enzyme to Carma1 and therefore directly involved in the regulation of Carma1 phosphorylation. Such direct interactions of the constant regulatory PP2A subunit has also been observed for other substrates, for example the TNF α signalling adapter TRAF2 and the NF- κ B family member p65 (Yang *et al*, 2001; Li *et al*, 2006).

Previous studies have proposed various functions of PP2A in NF- κ B signalling. It was shown that the activity of the IKK complex is influenced by PP2A, but it remains controversial whether PP2A is actually exerting an activating or inhibiting effect on the IKK complex (DiDonato *et al*, 1997; Kray *et al*, 2005; Palkowitsch *et al*, 2008; Witt *et al*, 2009). T-cell-specific deletion of the IKK β kinase MEKK3 impairs NF- κ B activation after TCR/CD28 stimulation (Shinohara *et al*, 2009) and overexpression analyses suggested that MEKK3 may be targeted by the catalytic PP2A subunit PPP2CA, but it remains to be investigated whether PP2A is directly involved in controlling MEKK3 phosphorylation and activity in T cells (Sun *et al*, 2010). Furthermore, PP2A can associate with the CD28 co-stimulatory receptor in T cells and based on OA inhibition, it was postulated that PP2A inhibits co-receptor signalling (Chuang *et al*, 2000). However, these analysis were exclusively based on OA inhibition, which was also shown to effectively inhibit PP4 (Zolnierowicz, 2000). Deletion of PP4 in the T-cell lineage causes defects in T-cell development and pre-TCR signalling (Shui *et al*, 2007).

By showing that Carma1 phosphorylation is enhanced in PPP2R1A knockdown T cells, we identified a new critical PP2A target for IKK/NF- κ B upstream signalling in T cells. Other PP2A-dependent mechanisms may certainly contribute to balance cytokine expression. An interesting observation has been made in T cells from systemic lupus erythematosus

(SLE) patients that have a decreased IL-2 production resulting from increased amounts and activity of the PP2A catalytic subunit PPP2CB (Katsiari *et al*, 2005). Knockdown of PPP2CB enhanced AP1 activity and promoted an increased binding of CREB to the IL-2 promoter, which was suggested to bring IL-2 production to normal levels in T cells from SLE patients, indicating that PP2A can also act at the level of IL-2 transcription. However, by showing that PP2A targets activated phospho-Carma1, we propose that the negative influence of PP2A on CBM/IKK/NF- κ B signalling may also contribute to dysregulation of IL-2 production and altered effector T-cell differentiation or function in an autoimmune disease like SLE.

Materials and methods

Antibodies, reagents, siRNAs and plasmids

The following antibodies were used: human CD3, human CD28, mouse IgG1 and mouse IgG2a, PP2A catalytic α (PPP2CA), PKC θ , IKK α (all BD Bioscience); Malt1 (B12), Bcl10 (331.3 (WB), C17 (IP)), Actin (I-19), Carma1 (N20 (IP)), HA (Y11), p65 (A) (all Santa Cruz Biotechnology); Flag-M2 (Sigma); Carma1 (1D12 (WB)), PP2A A subunit (PPP2R1A/B; #2039), PP2A C subunit (PPP2CA/B; #2038), phospho-Thr538 PKC θ , phospho-IKK α / β (all from Cell Signaling); p50 (Rockland); HA 12CA5 (Roche). Phospho-specific antibody against chicken Ser668/human Ser645 Carma1 (Shinohara *et al*, 2007). The following reagents and short interfering (si) RNAs (100 nM) were used: Phorbol 12-myristate 13-acetate (PMA) (200 ng/ml), Ionomycin (300 ng/ml) and OA (250 nM) (from Calbiochem); r-IL-2 (20 U/ml), protease inhibitor cocktail, anti-HA Affinity Matrix (all Roche); Protein G Sepharose (GE Healthcare); λ phosphatase (NEB); Dynal T-cell Positive Isolation Kit and Dynabeads (114.45) (Dynal Invitrogen). siGFP control, siPPP2R1A-1: TGAAGAAGCTAGTGGAAAA, siPPP2R1A-2: TGGACAACGTCAAG AGTGA, siPPP2CA: GATACAAATCTTGTGTTA (all Eurogentec). All tagged Carma1, PPP2R1A, PPP2CA wt and PPP2CA mut H118N; L199P (Evans *et al*, 1999; Myles *et al*, 2001) cDNAs were cloned in the pEF backbone vector (Invitrogen). Adenoviral vectors encoded shRNA sequences as a control: TTTTGGCCTTTTGTAGCTG and to target mouse PPP2R1A: GCACCGAATGACTACTCTT.

Cell culture

HEK293 cells were transfected using standard calcium phosphate precipitation protocols. Standard cell culture, transfection and stimulation of Jurkat T cells (P/I or CD3/CD28 antibody co-ligation) were performed as described (Wegener *et al*, 2006). For RNA interference, Jurkat T cells were transfected with 100 nM siRNA and Atufect transfection reagent (0.5–1 μ g/ml) (Silence Therapeutics, Berlin) and analysed after 72 h. JPM50.6 cells were transfected with 30 μ g DNA by electroporation using GenePulser Xcell (Bio-Rad). Mouse primary CD4⁺ T cells were isolated from spleen and lymph nodes using anti-mouse CD4 Dynabeads (Invitrogen). Isolated T cells were cultured in RPMI media (10% FCS, 0.1 mM β -mercaptoethanol, 100 U/ml penicillin-streptomycin, 10 mM HEPES). Stimulation was performed on goat anti-hamster IgG-coated plates adding anti-CD3 and anti-CD28 and anti-IL-4 (10 μ g/ml) as well as recombinant IL-12 (10 ng/ml) to induce Th1 differentiation. After 48 h, cells were transferred to a new plate and cultured with IL-2 (20 U/ml) for 72 h and another 24 h without IL-2 followed by re-stimulation for 6 h and by adding BFA (10 μ g/ml) for the last 2 h. Type 5 replication-deficient adenoviruses encoding shRNA against PPP2R1A or control shRNA were purchased (Sirion Biotech) and amplified in HEK293 cells. Titters were determined in A549 cells by FACS 40 h after transduction. Primary CD4 T cells from coxsackie adenovirus receptor transgenic mice (Wan *et al*, 2000) were transduced with recombinant viruses at an MOI of 100 o/n or incubated at an MOI of 300 for 1 h, respectively. Transduction efficiency was determined by FACS.

Co-immunoprecipitation

For co-IP studies, Jurkat T cells were lysed in 900 μ l co-IP buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM DTT, 10 mM sodium fluoride, 8 mM β -glycerophosphate, 300 μ M sodium vanadate and protease inhibitor cocktail). For co-IPs

in HEK293 cells a Triton X-100 containing co-IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail) was used. IPs were performed by adding antibodies against Bcl10, Carma1, PPP2R1A/B, IKK α , Flag-M2 or anti-HA.

Quantitative RT-PCR

LightCycler real-time PCR analysis using LC-480 SybrGreen PCR mix (Roche) was done with cDNA generated from DNA-free RNA samples. cDNAs were reverse transcribed from 0.7 μ g total RNA and random hexamers with the Superscript II kit (Invitrogen) according to the manufacturer's protocol. Cycling conditions on a Roche LightCycler 480 (LC-480) was performed using a standard LightCycler protocol. Relative quantification of IL-2 transcripts was carried out with housekeeping genes as calibrators for normalization (Radonic *et al*, 2004). The relative expression ratio was calculated from the real-time PCR efficiencies and the crossing point deviations of the target gene (IL-2) versus the housekeeping gene RNA polymerase II (RPII), as previously described (Pfaffl, 2001). Primers: IL-2 forward primer 5'-CACAGCTACAACTGGAGCATTTAC-3'; IL-2 reverse primer 5'-TGCTGATTAAGTCCCTGGGTC-3'.

EMSA, ELISA and luciferase assays

For electrophoretic mobility shift assay (EMSA), cells were lysed in whole-cell lysis buffer (20 mM HEPES pH 7.9, 350 mM NaCl, 20% Glycerin, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 1 mM DTT, 10 mM sodium fluoride, 8 mM β -glycerophosphate, 300 μ M sodium vanadate and protease inhibitor cocktail). NF- κ B EMSA were performed essentially as described previously (Duwel *et al*, 2009). For IL-2 ELISA, cell supernatant of Jurkat T cells was measured with the human IL-2 ELISA kit (eBioscience). Luciferase reporter assays from JPM50.6 and Jurkat T cells were done after electroporation with NF- κ B reporter plasmid 6 $\times$ NF- κ B B luc pGL2 (Firefly luciferase) and pTK luc (Renilla luciferase). At 72 h post-transfection, cells were stimulated with P/I for 4 h. Luciferase activity was measured using the dual luciferase reporter kit (Promega).

In vitro phosphatase assay

CBM complex IP in Jurkat T cells was performed as described above. The precipitated CBM was incubated with λ phosphatase 30°C for 20 min. Controls were performed with heat-inactivated

λ phosphatase or without phosphatase. For dephosphorylation of Carma1 using recombinant PPP2CA, cells were lysed in co-IP buffer without phosphatase inhibitors. After Carma1 IP, the precipitates were incubated with 0.2 or 0.4 μ g recombinant PPP2CA (Cayman Chemical) at 37°C for 25 min in a reaction buffer containing 40 mM Tris (pH 8.4), 34 mM MgCl₂, 4 mM EDTA, 2 mM dithiothreitol, 0.05 mg/ml BSA. For dephosphorylation using transfected PPP2CA wt and mut (H118N; L199P), cells were lysed in co-IP buffer. Endogenous Carma1 and overexpressed HA-tagged PPP2CA wt or mutant were immunoprecipitated with anti-Carma1 and anti-HA antibody. Precipitates were washed three times to remove phosphatase inhibitors before incubation with the reaction buffer as outlined above.

FACS sorting and intracellular IL-2 and IFN γ analysis by flow cytometry

Single-cell solutions were stained with fixable life-dead stain (Invitrogen), fixed in 4% PFA and permeabilized in PBS/0.5% Saponin/1% BSA. Unspecific binding was blocked with anti-FCR3-IgG3 and staining was performed with fluorescence-labelled anti-IFN γ or anti-IL-2 (eBioscience). FACS data acquisition were performed on a FACS Calibur device or LSR2 (Becton Dickinson) or were sorted on a FACS Aria II (Becton Dickinson) and raw data were analysed with Flow Jo (Treestar) software.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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