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Phosphoinositide-dependent kinase 1 integrates T cell receptor and CD28 co-receptor signaling to effect NF-κB induction and T cell activation

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Abstract

In addition to T cell receptor (TCR) ligation, activation of CD28 coreceptor by costimulatory molecule B7 is required for transcription factor NF-κB induction and robust T cell activation, though exactly how CD28 contributes to this remains incompletely understood. We demonstrated here that phosphoinositide-dependent kinase 1 (PDK1) plays an essential role in integrating TCR and CD28 signals. Upon deletion of PDK1 in T cells, TCR-CD28 signals failed to induce NF-κB activation or protein kinase C θ (PKC-θ) phosphorylation, although T cell survival and pathways dependent on p38 and Jnk kinases or transcription factor NF-AT were unaffected. CD28 facilitated NF-κB activation by regulating PDK1 recruitment and phosphorylation, which are necessary for efficient binding of PDK1 to PKC-θ and CARMA1, and thus for NF-κB induction.

INTRODUCTION

Engagement of the T cell antigen receptor (TCR) with foreign antigen-major histocompatibility complex (MHC) activates $CD4^+$ helper T cells through several different signaling pathways. These pathways induce proliferation of activated T cells and promote their further differentiation into effector T_H1 and T_H2 and memory cells1. CD28 (A000548) coreceptor ligation, along with the TCR, is required for full activation of T cells2,3. CD28 coreceptor ligation was shown to be required for efficient activation of NF-κB3, NF-AT4, AP-15, and for production of cytokines that are regulated by these transcription factors. Consequently, the immune response to foreign antigen is severely diminished in CD28-

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deficient (*Cd28*$^{-/-}$) mice6,7. The key downstream effector of CD28 ligation is phosphatidyl inositol 3-kinase (PI3K) and although pharmacological and genetic studies have demonstrated the importance of this kinase in T cell activation8, it still unclear whether CD28 triggers a distinct signaling cascade or whether signals from CD28 and PI3K synergize with pathways that emanate from the T cell receptor.

Phosphoinositide-dependent kinase 1 (PDK1, A001740), a pleckstrin-homology domain (PH-domain) containing protein kinase, is a well-characterized downstream effector of PI3K that has been suggested to act as a master regulator of the protein kinase A-protein kinase G-protein kinase C (AGC) family of kinases9. Upon activation, PI3K converts the membrane phospholipids, phosphatidyl inositol bisphosphate (PIP_2), into phosphatidyl insositol trisphosphate (PIP_3) through phosphorylation10. PIP_3 then functions as a secondary messenger for proteins containing PH domains10. It is currently believed that PI3K does not affect PDK1 through direct phosphorylation; rather through generation of PIP_3, PI3K induces PDK1 relocalization to phospho-lipid enriched membrane fractions9,11. The mechanism by which PDK1 is activated remains unclear, and some reports have claimed that PDK1 is constitutively active9,11. Several serine and threonine residues in PDK1 are reportedly phosphorylated12,13, although the consequence of these post-translational modifications and the kinase(s) responsible remain to be defined. In any case, it is known that activated PDK1 can phosphorylate substrate kinases such as AKT, which through its own PH domain also colocalizes to PIP_3 enriched membranes. Other PDK1 substrates such as PKCs (protein kinase C) do not have PH domains, and hence their co-localization to PDK1 is likely through as yet uncharacterized mechanisms9.

The kinases activated by PDK1 are important for cell metabolism, survival, and activation, and hence it is perhaps not surprising that PDK1 knockouts exhibit early embryonic lethality14. In the immune system, conditional deletion of PDK1 in developing thymocytes blocks T cell development at double-negative (DN) 4 stages through prevention of DN4 thymocyte proliferation15. However, the inability to isolate PDK1-deficient mature T cells has prevented the analysis of PDK1 function in T cell activation. In Jurkat T cells, using shRNA, it has been demonstrated that PDK1 plays a critical role in T cell activation by nucleating the assembly of a signaling complex in response to CD28 engagement, thereby leading to activation of NF-κB16. However the lack of PTEN, a critical PI3K regulator, in Jurkat T cells has led to questions about the relevance of these findings to primary T cells. In addition a controversy about the status of protein kinase C-θ(PKC-θ) phosphorylation has led to further confusion surrounding the role of PDK1 in T cell receptor and CD28-mediated NF-κB activation16.

In the current study, using a mouse with a T cell specific deletion of PDK1, we find that while TCR-CD28-mediated NF-κB activation is abolished in the absence of PDK1, other signaling pathways such as p38 mitogen-activated protein kinase (MAPK), Jun kinase (Jnk), and NF-AT were not significantly affected. In addition, somewhat surprisingly we find that survival of $CD4^+$ T cells is not affected even though PDK1 is thought to be important for cell growth and metabolism. The studies in this report allow us to propose that CD3 ligation primarily regulates PKC-θ localization while CD28 ligation is required for PDK1 localization; CD28 ligation leads to PDK1 phosphorylation on T513 through a PI3K-

dependent pathway; and the phosphorylation of PDK1 at T513 is crucial for PDK1 to bind both PKC-θ (A001933) and the scaffold and membrane-associated guanylate kinase (MAGUK) family member CARMA1 (A003863), which are essential for NF-κB activation. Therefore these studies provide convincing genetic evidence for the important role of PDK1 in integrating signals from the TCR and CD28 leading to the activation of NF-κB.

RESULTS

CD4$^+$ T cell activation requires PDK1

To better clarify the actual role of PDK1 in primary T cells, we used mice in which the *PDK1* gene was flanked by loxP sites, and then crossed these mice with transgenic mice expressing Cre under the control of the *Cd4* promoter (CD4-Cre) to delete *Pdk1* in developing T cells from double-positive (DP) stage of T cell development. Unlike *Pdk1* deletion by Lck-Cre (*Cre* gene expression under *Lck* promoter) that led to a block in thymocyte development between DN3 and DN4 stages15, deletion of *Pdk1* by CD4-Cre at the DP stage does not significantly affect CD4$^+$ T cell development (Fig. 1a,b) even though these cells have dramatically reduced PDK1 protein level compared to wild-type littermate control (Fig 1c). Moreover, total thymocyte numbers as well as total lymph node T cell numbers were not significantly altered. The proportion of CD4$^+$ T cells was slightly decreased than that in wild-type littermate control mice in both peripheral blood and lymph nodes. Surprisingly, however, we found that the number and proportion of CD8$^+$ T cells were dramatically reduced in the thymus and this reduction in numbers was maintained in the periphery (Fig. 1a,b). This effect could be a consequence of PDK1 deletion in DP thymocytes that for unknown reasons disproportionately affects CD8$^+$ T cell development. In addition, we found that other Thy1$^+$ cell types including γδ T cell and NK T cell are increased in the periphery (our unpublished observations). We isolated mature CD4 single-positive (SP) thymocytes by FACS and stimulated the cells with anti-CD3 + anti-CD28 (anti-CD3–anti-CD28) and found that up-regulation of CD25 and production of interleukin 2 (IL-2) were dramatically impaired in the absence of PDK1 (Fig. 1d,e).

CD4$^+$ T cell survival does not require PDK1

As PDK1 has been implicated in cell survival signaling pathways, e.g. those involving AKT, we wanted to compare the survival kinetics of PDK1-deficient CD4SP thymocytes and peripheral CD4$^+$ T cells. We therefore analyzed the population of dead cells by staining with 7AAD and ANX-V after culturing the thymocytes for 3 or 6 days, with or without IL-7. This experiment showed that survival of mature CD4SP thymocytes was not significantly reduced in PDK1 deficient cells, and survival of PDK1-deficient mature thymocytes was enhanced by IL-7 treatment, suggesting that PDK1 was not involved in IL-7-mediated survival of thymocytes (Fig. 2a). However, this experiment was performed in *in vitro* culture conditions and therefore to address the role of PDK1 in T cell survival *in vivo* we generated mice bearing the congenic markers Thy1.1 and Thy1.2. Transferring wild-type or PDK1-deleted CD4 cells into recipient mice showed no difference in survival of wild-type vs. PDK1-deleted CD4$^+$ T cells (Fig. 2b). In addition, ANX V-positive apoptotic cells in lymph nodes was not significantly different between the wild-type and knockout animals (data not shown). Therefore these data strongly suggested that PDK1 is not essential for resting CD4$^+$

T cell survival. To test the role of PDK1 in the survival of $CD8^+$ T cells, we isolated $CD8^+$ T cells from *Pdk1*$^{flox/flox}$ or *Pdk1*$^{+/+}$ mice and then infected them with Cre-expressing retroviruses. We found that survival of $CD8^+$ T cells, similar to $CD4^+$ T cells, was not significantly affected by knocking out PDK1 (Fig. 2c), even though the number of $CD8^+$ T cells is dramatically decreased in the CD4-Cre PDK1-deficient mice (Fig. 1a,b). This suggests that the reduction of $CD8^+$ T cells seen is most likely due to a block in the development of these cells.

TCR-CD28-mediated NF-κB activation requires PDK1

TCR and CD28 co-ligation results in activation of multiple pathways, including those leading to NF-κB, NF-AT, Jnk, and p38 MAPK17,18. We therefore examined the status of all these pathways following TCR and CD28 coreceptor ligation in primary $CD4^+$ T cells isolated from lymph nodes of CD4-Cre–*Pdk1*$^{flox/flox}$ or CD4-Cre–*Pdk1*$^{+/flox}$ Mice. Receptor ligation-induced IκBα degradation (Fig. 3a) and NF-κB DNA binding activity (Fig. 3b) were strongly inhibited in the PDK1-deficient cells, despite relatively normal phosphorylation of Jnk and p38 MAPK (Fig. 3a), and activation of NF-AT (Fig. 3b). Consistent with our previous findings, induced phosphorylation of PKC-θ at threonine T538 was also strongly impaired in the absence of PDK1. In fact the pattern of signaling defects resembles that reported for mature T cells from PKC-θ-deficient (*Pkcq*$^{-/-}$) 20. PKC-θ-deficient cells are defective in NF-κB activation but not other signaling pathways such as Jnk, p38 MAPK and NF-AT19 (Supplementary Fig. 1 online). Interestingly, the level of PKC-θ was slightly decreased in PDK1-deficient $CD4^+$ T cells suggesting that PDK1 might be involved in the stability of PKC-θ in activated cells (Fig. 3a). We also examined the status of phosphorylation of AKT, another substrate of PDK1. Phosphorylation of T308 in the activation loop of AKT was impaired upon deletion of PDK1, but phosphorylation of S473 in the hydrophobic region was not affected (Supplementary Fig. 2 online). Expression of IL-2, one of the key NF-κB-dependent target genes in T cells, was also dramatically reduced in PDK1-deficient primary $CD4^+$ T cells (Fig. 3c). The reduction of IL-2 however, was not due to destabilization of IL-2 mRNA (Supplementary Fig. 3 online). Whereas PDK1 did not significantly affect survival, proliferation of $CD4^+$ T cells was significantly reduced in the absence of PDK1 upon stimulation with anti-CD3–anti-CD28 (Fig. 3d).

To address the concern that mature T cells that develop in the absence of PDK1 might be somehow different from normal T cells, we also deleted PDK1 in mature T cells from *Pdk1*$^{flox/flox}$ mice by transducing the T cells with a Cre-expressing recombinant retrovirus (Fig. 3e,f and Supplementary Fig. 4 online). The PDK1-deleted $CD4^+$ T cells, detected by IRES driven GFP expression, fail to upregulate the activation marker CD69, further implicating PDK1 in TCR-CD28-mediated T cell activation (Fig. 3e).

In light of recent data demonstrating differences in TCR signaling to NF-κB in naïve and effector T cells we wanted to assess the role of PDK1 in effector T cells. This is an important question as kinase TAK1 (A002249), a critical regulator of cytokine induced NF-κB pathways, is required for TCR-mediated signaling in naive T cells, whereas in effector T cells it participates in cytokine-mediated survival signaling but not TCR signaling to NF-κB17. However, as a result of the inability of *Pdk1*$^{-/-}$ cells to signal to NF-κB, deletion of

PDK1 during T cell development leads to a $CD4^+$ T cell population that cannot become effector cells. We therefore isolated splenic $CD4^+$ T cells from *Pdk1*$^{flox/flox}$ or *Pdk1*$^{+/+}$ mice, and activated the isolated cells with concanavilin A (ConA) to convert them into $CD25^{high}CD44^{high}$ effector T cells (data not shown). The cells were then infected with Cre-expressing retroviruses to delete *Pdk1*. The PDK1-deficient effector T cells, like PDK1-deficient naive T cells, failed to activate NF-κB, but not JNK or p38 MAPK in response to TCR stimulation (Fig. 3f). IL-2 production was also dramatically inhibited in PDK1-deficient effector $CD4^+$ T cells (Supplementary Fig. 4 online). Unlike naive cells, the basal level of T538 phosphorylation is significantly higher in effector T cells. Therefore not surprisingly, the relative increase in T538 phosphorylation upon stimulation is less than that in naive cells. The higher level of basal PKC-θ phosphorylation is however dependent on PDK1 (Fig. 3f). It is known that effector T cells are less dependent on CD28 signals for activation hence it is likely that effector T cells compensate for the requirement for CD28 during NF-κB activation by maintaining a constitutive PDK1-PKC-θ activation cascade.

To further establish the role of PDK1 in effector T cells, we crossed *Pdk1*$^{flox/flox}$ mice with transgenic mice expressing Cre from an estrogen receptor dependent cassette. Effector T cells isolated from these mice were treated with tamoxifen to delete *Pdk1*. These PDK1-deficient cells show the same pattern of signaling defects: failure to activate NF-κB or produce IL-2, but displaying normal JNK and MAPK activation (Supplementary Fig. 5 online). In addition, further analysis of these cells revealed that PDK1 was dispensable for both intrinsic survival, as well as IL-2-dependent survival (Supplementary Fig. 6 online). Therefore, PDK1 functions in TCR-induced NF-κB activation in both naïve and effector T cells.

CD28 regulates PDK1 localization and phosphorylation

The TCR complex and CD28 co-localize at the central supra-molecular activation cluster (cSMAC)20 upon stimulation of T cells, and this co-localization is necessary for strong NF-κB activation3 (Supplementary Fig. 7 online). We found that PDK1 and PKC-θ also co-localize at the immunological synapse (Fig. 4a and Supplementary Fig. 8 online) and they can be co-immunoprecipitated following anti-CD3–anti-CD28 stimulation of both Jurkat T cells (Fig. 4c) and primary $CD4^+$ T cells (Supplementary Fig. 9 online). Intriguingly, despite the interaction between PDK1 and PKC-θ observed following anti-CD3–anti-CD28 stimulation, recruitment of PDK1 and PKC-θ can occur independently. That is, treatment with anti-CD3 alone leads to PKC-θ recruitment, while treatment with only anti-CD28 leads to PDK1 recruitment (Fig. 4a). However, engagement of both CD3 and CD28 is required for induced phosphorylation of PKC-θ (Fig. 4c and Supplementary Fig. 10 online). CD3 and CD28 co-stimulation also increased levels of PDK1 and PKC-θ recruitment (Fig. 4a). Therefore, TCR and CD28 co-receptor ligation independently induce the recruitment of PKC-θ and PDK1, respectively. These data indicate that T cell co-receptor ligation is crucial for recruitment of PDK1 to the cSMAC, yet they do not address whether PDK1 kinase activity is also regulated by CD28 signaling. Interestingly, besides the localization of PDK1 and PKC-θ to the cSMAC, small amounts of both PDK1 and PKC-θ could be detected at the region opposite to the cSMAC, which is referred to as the anti-synapse. However the significance of this localization remains unclear.

CD28 receptor ligation induces PI3K activation and the activated PI3K converts the phospholipid PIP_2 into PIP_3, which regulates PDK1 function, although the mechanism remains somewhat controversial. One group has reported that PDK1 kinase activity is not changed upon signaling and the status of PDK1 phosphorylation also remains unchanged9,21. However, others have reported that PDK1 is phosphorylated upon signaling12,13 and that PIP_3 can induce PDK1 autophosphorylation *in vitro* leading to increased PDK1 activity22. In our studies, anti-CD3–anti-CD28 stimulation does increase PDK1 kinase activity (Fig. 4b). Furthermore, we find that, while PIP_3 increases PDK1 kinase activity, PIP_2 strongly inhibits PDK1 kinase activity *in vitro* (Fig. 4b). Moreover, both CD28 and CD3-CD28 receptor ligation induce the appearance of a slower migrating form of PDK1 (Fig. 4c & Supplementary Fig. 10 online). This slower migrating band represents a phosphorylated form of PDK1 as treatment with phosphatase reverses the shift in mobility (Supplementary Fig. 11 online). Serine 241 in PDK1 has been shown to be phosphorylated, however it is not the site of CD3-CD28 induced phosphorylation as a serine 241 phospho-specific antibody detects PDK1 in unstimulated cells (Fig. 4c). To test whether the TCR-CD28-mediated PDK1 phosphorylation is dependent on PI3K, we analyzed PDK1 phosphorylation in cells treated with the PI3K inhibitors wortmannin, LY294002, and PI3Kγ-inhibitor II. Remarkably, PDK1 phosphorylation was inhibited by wortmannin and LY294002 (Fig. 4d). Furthermore, PKC-θ phosphorylation and IκBα degradation, which we have shown are dependent on PDK1, are also blocked by wortmannin and LY294002 (Fig. 4d). However, PI3Kγ-inhibitor II does not affect PDK1 phosphorylation, PKC-θ phosphorylation and IκBα degradation (Fig. 4d). The same pattern was seen in IL-2 production in mouse T cells, which was inhibited by wortmannin and LY294002, but not by PI3Kγ-inhibitor II (Fig. 4e). Therefore it seems that the gamma subunit of PI3K is not involved in TCR-CD28-mediated NF-κB activation and suggests strongly that PI3K-mediated PDK1 phosphorylation is directly linked to TCR-CD28-mediated NF-κB activation. Similarly, a previous report has shown that mutation of the PI3K binding site on CD28 blocks NF-κB activation23.

Phosphorylation of PDK1 at threonine 513

Both here and in earlier studies16, we have demonstrated that PDK1 binds to PKC-θ in primary T cells (Supplementary Fig. 9 online) and Jurkat T cells (Fig. 4c). Moreover, PDK1 can induce weak PKC-θ phosphorylation *in vitro*, which correlates with the relatively weak binding between these proteins under *in vitro* conditions. The weak PDK1–PKC-θ interaction observed *in vitro* and by overexpression, and the correlation between CD28-induced PDK1 phosphorylation and PKC-θ co-immunoprecpitation, suggested that induced PDK1 phosphorylation might augment PDK1–PKC-θ interactions. We, therefore, hypothesized that TCR-CD28-mediated PDK1 phosphorylation is an important regulatory step for the induction of PKC-θ-mediated NF-κB activation. To test this hypothesis we sought to identify the PDK1 phosphorylation site that affects the binding between PDK1 and PKC-θ. We chose to analyze 11 PDK1 phosphorylation sites, which had been identified previously by other groups, and systematically assessed their contribution to PKC-θ binding by mutating them to alanines. We then carried out binding analysis between PKC-θ and all 11 PDK1 mutants, and found one, the T513A mutant, was most dramatically affected in its binding to PKC-θ (Supplementary Fig. 12 online). Alignment of sequences surrounding

T513 with PDK1 sequences from other species indicates that T513 is highly evolutionarily conserved. Surprisingly, this site is also conserved in AKT family members (Fig. 5a and Supplementary Fig. 13 online). Upon overexpression, wild type PDK1 binds weakly to PKC-θ (Fig. 5b) and it does not significantly increase the level of NF-κB activation induced by PKC-θ-CARMA1-Bcl10-Malt1 (θCBM) overexpression (Fig. 5d). This data indicates that T513 phosphorylation is tightly regulated by upstream signals because overexpression of wild type PDK1 itself is not enough to allow efficient binding to PKC-θ and CARMA1, and hence activation of the θCBM-mediated NF-κB activation. However, introduction of a phosphomimetic aspartic acid or glutamic acid residue at T513 (T513D or T513E) leads to both dramatically enhanced binding to PKC-θ (Fig. 5b) and increased NF-κB activation induced by θCBM overexpression (Fig. 5d).

It was unclear from these data however, how phosphorylation of PDK1 at T513, well outside the kinase domain, might regulate PDK1 function. Interestingly changing T513 into alanine induces strong binding between PKC-θ and PDK1 (Fig. 5b), but does not increase NF-κB activation by θCBM overexpression (Fig. 5d). These data suggest that while structural properties of the protein around T513 regulate efficient binding to PKC-θ, a negative charge at this site (either through phosphorylation or substitution with phosphomimetic amino acids) is specifically required for proper NF-κB activation. This discrepancy between binding of PDK1 to PKC-θ (by changing threonine 513 to either aspartic acid or alanine) versus ability of overexpressed PDK1 to activate NF-κB (seen only by changing threonine 513 to aspartic acid) could be explained by the effect of these mutations on interaction of PDK1 with CARMA1. Indeed, we find that efficient binding between PDK1 and CARMA1 is seen only upon introduction of a negative charge at position 513 (Fig. 5c). Therefore, while the T513A mutation can lead to strong binding between PDK1 and PKC-θ, it does not induce strong NF-κB activation because it does not efficiently recruit CARMA1. Therefore, CD28-regulated PDK1 phosphorylation plays a central role in PKC-θ–CARMA1-mediated NF-κB activation. Interestingly, we also found that PDK1 is hyperphosphorylated upon PKC-θ binding (Fig. 5b) and not following CARMA1 binding (Fig. 5c). We also found that this hyperphosphorylation is linked to PKC-θ kinase activity although the exact mechanism remains to be explored.

T513 phosphorylation is critical for CD4$^+$ T cell activation

To test whether PDK1 is actually phosphorylated upon TCR-CD28 ligation, we carried out whole cell phospho-labeling of primary T cells, followed by immunoprecipitation of PDK1 (Fig. 6a, upper panel). In parallel we immunoblotted the samples with a T513-phospho specific antibody. As shown in the upper two panels of Figure 6a, treatment of T cells with anti-CD3–anti-CD28 leads to phosphorylation of PDK1 on T513. To demonstrate that this phosphorylation of PDK1 was dependent on CD28, we stimulated T cells with either anti-CD3, anti-CD28, or anti-CD3–anti-CD28, and followed T513 phosphorylation by immunoblotting with a T513 phospho-specific antibody. As shown in Figure 6b, T513 phosphorylation is observed only upon CD28 engagement. However, as seen in the Fig. 4a, CD28-ligation alone is unable to efficiently colocalize PDK1 and PKC-θ, strongly suggesting that signals from the TCR synergize with signals from CD28 to activate both PDK1 and PKC-θ, and result in NF-κB activation. While total PDK1 phosphorylation

continues to increase until 60 min following stimulation, T513 phosphorylation decreases slightly between 15 to 60 min (Fig. 6a). This suggests the possible existence of other CD3–CD28-inducible phosphorylation sites on PDK1, even though T513 is the most critical for NF-κB activation.

To test more directly whether T513 of PDK1 is important for TCR-CD28-mediated T cell activation we performed reconstitution of PDK1 deficient T cells with different PDK1 mutants. While NF-κB activation and IL-2 production following TCR-CD28 ligation are strongly impaired in PDK1 knockouts, these defects can be rescued upon introduction of wild type PDK1 using recombinant lentivirus (Fig. 6c). Remarkably, the introduction of PDK1 T513D leads to more IL-2 production compared to wild type PDK1. This is likely because the wild-type PDK1 is not phosphorylated in the absence of upstream signal, whereas the T513D mutant mimics the phosphorylated protein, thus showing activity. Conversely, if there are upstream signals, wild type PDK1 can be phosphorylated at the T513 site. However, T513D cannot be further phosphorylated at the site during stimulation. Only T513D localization is redirected by CD28 ligation. Therefore, the increase of IL-2 level by mutant T513D is limited compared to wild type PDK1. Moreover, introduction of the PDK1 T513I mutant, which is defective in binding to PKC-θ and CARMA1 and NF-κB activation, and which cannot be phosphorylated on T513 upon TCR-CD28 signaling, fails to rescue the defect in IL-2 production following TCR-CD28 receptor ligation. As we need phosphorylation of PDK1 to get strong interaction with PKC-θ, in the transfection experiment in Figure 5d, where we transfected PDK1 constructs into 293 cells, the wild-type PDK1 is unphosphorylated and hence behaves similarly to the T513I mutant. Complementation with the PDK1 T513D mutant can replace CD28 signaling for IL-2 production (Fig. 6c), strongly suggesting that it is CD28 signaling that leads to PDK1 activation and phosphorylation. However the induction of IL-2 in TCR stimulated PDK1 T513D reconstituted cells is augmented by anti-CD28, which indicates that both proper localization and T513 phosphorylation of PDK1 are required for efficient signaling. Alternatively, synergism between CD28 and CD3 for activation of other TCR-induced signaling pathways that are crucial to IL-2 transcription, e.g. NF-AT, may explain this difference. The data presented above therefore provide strong support for the hypothesis that CD28-induced phosphorylation of PDK1 threonine 513 is important for PDK1 regulation during TCR-CD28 signaling.

DISCUSSION

Activation of NF-κB in T cells has been studied intensely and has revealed the involvement of a number of unique components in this signaling pathway24. These include PKC-θ and the CBM complex. The link between these components and the IKK complex is an area of active investigation and interest; however the mechanism by which the antigen receptor itself engages this pathway has remained relatively poorly studied. In particular a well-established fact about NF-κB activation in T cells has received very little attention, namely the obligatory co-engagement of CD28 along with the TCR to generate robust NF-κB activation. A reason for this dearth of attention has been the lack of information about the signaling pathways triggered by CD28, and in particular the confusion about how distinct CD28 signals are integrated with the well-characterized signals from the T cell receptor. The

studies in this paper provide convincing evidence that PDK1, a critical downstream molecule in the CD28–PI3K pathway, is the key molecule that acts to integrate the TCR and CD28 signaling pathways, thereby allowing their synergistic action in the activation of NF-κB. Based on our experiments, we propose the following model of TCR-CD28 signaling to NF-κB: Initially, engagement of the T cell receptor complex with antigen-MHC leads to CD28 recruitment to the cSMAC region and consequent engagement with B7 molecules. Activated TCR signaling complexes recruit PKC-θ, while CD28, through PI3K-generated PIP_3, recruits PDK1 leading to conformational changes induced by auto-phosphorylation of PDK1 at T513. The phosphorylated PDK1 is then able to efficiently bind to both PKC-θ and CARMA1. The binding between PDK1 and PKC-θ induces PKC-θ activation loop phosphorylation, and the active PKC-θ then leads to induced phosphorylation of CARMA125–27. Phosphorylated CARMA1 undergoes a conformational change that allows it to recruit and assemble the CBM complex, and subsequently facilitate activation of IKK and NF-κB. NF-κB activation is dependent on PDK1, whereas other TCR-CD28-mediated pathways including JNK, p38 MAPK, and NF-AT are not.

A key finding in this report is the identification of T513 as a critical site whose phosphorylation is necessary to allow PDK1 to efficiently interact with both PKC-θ and CARMA1. The ability of PIP_3 to induce phosphorylation of PDK1 *in vitro* strongly suggests that T513 phosphorylation is an autocatalytic event22. Comparison of sequences between different related kinases, including members of the AKT family, reveals a conserved threonine positioned equivalently, suggesting that similar auto-phosphorylation events might be a general property of PI3K activated protein kinases. Remarkably, reconstitution of PDK1 deficient T cells with a mutant form of PDK1 that contains a phosphomimetic amino acid substitution at this site, allows activation of NF-κB in these cells by TCR engagement alone strongly supporting the importance of this phosphorylation in PDK1 regulation. The phosphorylation at T513 likely helps to maintain the PDK1 in a conformation that is triggered by PIP_3, but more importantly is critical for efficient recruitment of both PKC-θ and CARMA1. The presumed conformation change is however not sufficient for NF-κB activation, since a T513A mutant, which is also able to bind efficiently to PKC-θ, cannot efficiently activate NF-κB, because it cannot efficiently recruit CARMA1. Therefore T513 phosphorylation is the critical step that allows PDK1 to interact efficiently with both PKC-θ and CARMA1, thereby allowing robust NF-κB activation.

An important finding in this report is that survival of T cells is not significantly affected by PDK1 knockout, but PDK1 is required for T cell activation and proliferation. Moreover, TCR-CD28-mediated activation does not induce rapid apoptosis of PDK1-deficient, activated $CD4^+$ T cells. Upon prolonged stimulation, $CD4^+$ T cell survival is not significantly affected by the absence of PDK1 within the first 48 hours. However, stimulation for longer time periods (96 hours) leads to a greater loss of PDK1-deficient T cells compared to wild-type cells. This is most likely due to reduced IL-2 production in PDK1 knockout $CD4^+$ T cells, as supplementation with exogenous IL-2 can partly reverse the loss of stimulated cells. The incomplete rescue is probably due to reduced CD25 expression on the stimulated PDK1 deficient $CD4^+$ T cells.

Interestingly, although it is well known that PDK1 activates AKT through activation loop phosphorylation, studies using genetic knockouts have revealed differential requirement for PDK1 and AKT in thymocyte survival28. Whereas AKT1-AKT2 double knockout severely affects thymocyte survival29, knockout of PDK1 at an early stage of thymocyte development affects only proliferation of DN4 stage thymocytes, rather than their survival15. Therefore at present it is reasonable to assume that there is an alternate pathway for AKT activation, probably via phosphorylation of S473 by mTOR, that allows survival of PDK1 knockout thymocytes and mature $CD4^+$ T cells.

In summary, the findings in this report provide definitive genetic and molecular evidence for the critical role for PDK1 in activation of NF-κB in T cells in response to co-engagement of the TCR and CD28. In particular, PDK1 acts not only to directly interact with and activate PKC-θ, but also recruits CARMA1, thereby allowing PKC-θ to phosphorylate CARMA1, a critical step in the subsequent assembly of the CARMA1-Bcl10-MALT1 complex. A surprising finding of our studies was the apparent lack of dependence on PDK1 in the survival of unstimulated, mature T cells. Therefore the specific and restricted role of PDK1 in NF-κB activation in T cells, without significant effect on T cell survival, raises the possibility of developing PDK1-specific inhibitors to block T cell activation in autoimmune diseases and in transplantation.

METHODS

Mice and T cell isolation

Pdk1$^{flox/flox}$ mice30 were bred with CD4-Cre transgenic mice31 and offspring were bred with *Pdk1*$^{flox/flox}$ mice to generate CD4-Cre–*Pdk1*$^{flox/flox}$ and CD4-Cre–*Pdk1*$^{+/flox}$. *Pdk1*$^{flox/flox}$ mice were bred with ER-Cre transgenic mice (Cre-ER fusion protein expressing mice) and offspring were bred with *Pdk1*$^{+/flox}$ mice to generate ER-Cre–*Pdk1*$^{flox/flox}$ and ER-Cre–*Pdk1*$^{+/+}$ mice. PKC-θ–deficient (*Pkcq*$^{-/-}$) mice were received from Dr. Littman. All mice were kept in specific pathogen–free conditions in the animal care facility at Yale University (New Haven, Connecticut). All mouse experiments were approved by Institutional Animal Care and Use Committee of Yale University. $CD4^+$ T cells were isolated from spleen or lymphnode through negative isolation.

Cell culture and antibodies

HEK293 cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS). Jurkat T cells were maintained in RPMI1640 supplemented with 5% FBS. Anti-PDK1 antibody was purchased from Upstate. Anti-PKC-θ and anti-IκBα (C-21) were purchased from SantaCruz. Anti-Myc was purchased from Invitrogen. Anti-HA was purchased from Sigma. Anti-JNK was purchased from Cell signaling. Phosphoantibodies for IκBα at S32, JNK at T183,Y185, p38 MAPK at T180,Y182, PDK1 at S241 were purchased from Cell Signaling. Phosphoantibody specific for PKC-θ at T538 was purchased from BD Biosciences. Phosphoantibody specific for PDK1 at T513 was kindly provided by Dr. Harris. Anti-mouse CD3ε, anti-mouse CD28, anti-human CD3, and anti-human CD28 were purchased from BD Biosciences. Anti-mouse IgG was purchased from Sigma and anti-hamster IgG were purchased from GeneTex. APC-conjugated anti-mouse IL-2, PE-

conjugated anti-mouse CD4, PerCP-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD8α, PE-conjugated anti-mouse Thy1 and PE-conjugated anti-mouse B220 were purchased from eBiosciences.

Plasmids

pCDNA-Myc-PDK1 was constructed by insertion of myc sequence tagged-PDK1 open reading frame into the pCDNA3 vector. Mutagenesis of pCDNA-Myc-PDK1 was performed using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene). pEGZ-HA-PKC-θ was constructed by insertion of PKC-θ open reading frame (*Pkcq*) into pEGZ vector. pCDNA-CARMA1 and pCDNA-BCL10 were constructed by insertion of open reading frames of CARMA1 (*Card11*) and Bcl10 (*Bcl10*) into pCDNA3. Construction of pCDNA3-MALT1 was described previously32. pBIIx and pRenilla were described previously16.

Flow cytometry and Elisa analysis

Lymphocytes were isolated from the thymi, spleens, and lymph nodes of 6–8 week old mice. Cell viability was assessed by annexin V and 7-amino-actinomycin D staining according to the manufacturer's protocols (BD-Pharmingen). Stained cells were analyzed on a FACSCalibur. Intracellular IL-2 was analyzed with BD Cytofix-Cytoperm™ Plus and APC-conjugated anti-mouse IL-2 antibody. Secreated-IL-2 level was analyzed by IL-2 specific ELISA (ebioscience). Briefly, 1×10^4 cells per well were plated in 96 wells plates coated with anti-mouse CD3ε (5 μg/ml) and anti-mouse CD28 (5 μg/ml) and were incubated at 37 °C and 5% CO_2. 24 h later, culture medium was analyzed. In the case of lentiviral transduction, the remaining cells were used for analysis of V5-tagged PDK1 expression by intracellular staining with mouse anti-V5 and secondary donkey anti-mouse conjugated with Alexa 488 (Molecular Probes). Stained cells were analyzed on a FACSCalibur.

Retrovirus and lentivirus transduction

Recombinant retroviruses were produced in phoenix-Eco cell line by transfecting with pMSCV-Cre-IRES-GFP and recombinant retroviruses were harvested after 48 h. Total splenocytes were isolated from wild type mice or *Pdk1*$^{flox/flox}$ mice and were stimulated with ConA at 2 μg/ml for 24 h. After the stimulation, cells were infected with recombinant retroviruses having Cre gene using spin inoculation. Briefly, cells were pretreated with polybrene for 45 min and the medium was removed. The cells were then incubated with medium containing recombinant retroviruses and polybrene for 45 min and centrifuged for 90 min at 2,200 rpm. After centrifugation, virus-containing medium was removed and cells were cultured in fresh medium for the indicated time. Infected cells were analyzed by FACS or were sorted by Moflow or FACS aria based on GFP expression. Recombinant lentiviruses were produced following manufacturer's protocol (Invitrogen). Primary $CD4^+$ T cells were isolated from ER-Cre–*Pdk1*$^{+/+}$ or ER-Cre–*Pdk1*$^{flox/flox}$ mice through negative isolation (Miltenyi Biotech) and cells were incubated with IL-7. After 24 h, each recombinant lentivirus was introduced using the spin inoculation method described above. Infected cells were incubated with 4-hydoroxy-tamoxifen (OHT) (100 nM) for 4 d in media supplemented with IL-7.

In vivo cell labeling

Isolated $CD4^+$ T cells were washed with phosphate-free RPMI1640 medium two times and then cells were incubated with phosphate-free RPMI1640 containing 5% dialyzed FBS for 1 h at 37 °C supplemented with 5% CO_2. Cells were stimulated with plate bound anti-mouse CD3ε and anti-mouse CD28 for indicated time in the presence of 300 μCi of [^{32}P]orthophosphate per 3×10^6 cells. After labeling, cells washed with ice-cold PBS and endogenous PDK1 was immunoprecipitated. The immunoprecipitated PDK1 was analyzed by SDS-PAGE and autoradiography.

NF-κB luciferase assay

HEK293 cells (2×10^4) were plated in each well of a 12-well plate and were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). Total DNA amount was normalized using empty pCDNA3 vector. After 36 h, cells were lysed in 500 μl of TNT lysis buffer (50 mM Tri-HCl, pH7.9, 150 mM NaCl, and 1% (vol/vol) Triton X-100) for 10 min at room temperature. Debris was removed by centrifugation at 13,000rpm for 5min at room temperature. Luciferase and Renilla luciferase were measured using 10 μl of lysates respectively. Fold stimulation was calculated for each sample by dividing the luciferase activity, normalized to Renilla luciferase activity, by that observed in the sample containing only empty expression vector.

In vitro kinase assay

Endogenous PDK1 protein was immunoprecipitated from Jurkat T cell lysates with anti-PDK1 antibody. The immunoprecipitated PDK1 protein was incubated with 10 μCi of γ-[^{32}P]ATP for analysis of autophosphorylation activity of PDK1. *In vitro* kinase assay was performed in 1× PDK1 *in vitro* kinase assay buffer containing 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine and 1,2-dioleoyl-*sn*-glycerol-3-phospho-L-serine 22.

Coimmunoprecipitation

For pull-down analysis, Myc-tagged PDK1, PDK1 mutants and PDK1 deletion constructs and HA-tagged PKC-θ and PDK1-PH were used. To study molecular interactions, expression vectors were cotransfected into HEK293 cells by Lipofectamine 2000. Forty hours later, target proteins were immunoprecipitated with anti-HA, anti-PKC-θ, anti-PDK1, or anti-Myc antibody. The samples were subjected to SDS-PAGE and immunoblotting with anti-myc, anti-HA, anti-PKC-θ, or anti-PDK1. For endogenous interactions, 5×10^7 Jurkat or 1×10^7 primary $CD4^+$ T cells were used after stimulation with or without anti-CD3–anti-CD28. Cell lysates were immunoprecipitated with anti-PDK1. The samples were subjected to SDS-PAGE and immunoblotting with anti- PKC-θ or anti-PDK1.

Immunofluorescence microscopy

For immunofluorescence analysis, Jurkat T cells or primary $CD4^+$ T cells were incubated with dynabead M450 coated with anti-CD3, anti-CD28, or anti-CD3 + anti-CD28 (anti-CD3–anti-CD28) at a 1:1 ratio for 15 min at 37 °C. After the incubation, T cells conjugated with the dynabead were plated on poly-L-lysine-coated coverslips for 15 min at 37 °C. And then the cells were fixed with 4% paraformaldehyde (wt/vol) for 10 min at room

temperature, and permeabilized with permeabilization buffer (0.1% Triton X-100, 5% FBS in PBS, pH 7.5) for 10 min at room temperature. Cells were incubated with the indicated primary antibodies, anti-PKC-θ or anti-PDK1, for 30 min at room temperature and then incubated with either donkey anti-rabbit or anti-goat antibodies conjugated with Alexa 488 or Alexa 547 under identical conditions. Results were analyzed using either a Zeiss Axiophot microscope or by LSM510 confocal microscopy.

Nuclear extract preparation and electrophoretic mobility-shift analyses

Purified primary $CD4^+$ T cells (2×10^6) were washed in PBS and resuspended in 1 ml of hypotonic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1mM EDTA with protease inhibitors). The cells were incubated on ice for 15 min. Triton X-100 was added to a final concentration of 1% and then the cells were vortexed vigorously, incubated on ice for 10 min and centrifuged for 1 min. After centrifugation, supernatant was removed and pellet was resuspended with 10 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA and protease inhibitors). The tube was agitated for 30 min at 4 °C and the mixture centrifuged for 5 min. After centrifugation, supernatant was saved as a nuclear extract and glycerol was added to a final concentration of 20% (vol/vol); 3 μl of each extract was subjected to bandshift analysis. Electrophoretic mobility-shift analyses (EMSAs) were performed with the following oligonucleotides and binding buffers: NF-AT oligonucleotide: 5′-GCCCAAAGAGGAAAA TTTGTTTCATACAG-3′; NF-AT 5′ binding buffer: 50 mM Tris-HCl, pH 7.5, 500 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 50% glycerol, 250 mg/ml poly (dI:dC), 5 mM dithiothreitol and 1 mg/ml BSA; NF-κB oligonucleotide: 5′-ACCAAGAGGGATTTCACCT AAATC-3′; NF-κB binding buffer: 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mg/ml poly (dI:dC), 5 mM dithiothreitol and 1 mg/ml BSA. 5×10^5 c.p.m. of labelled probe was used in each reaction, and bandshifts were resolved on 5.5% polyacrylamide gels in 0.5 × TBE running buffer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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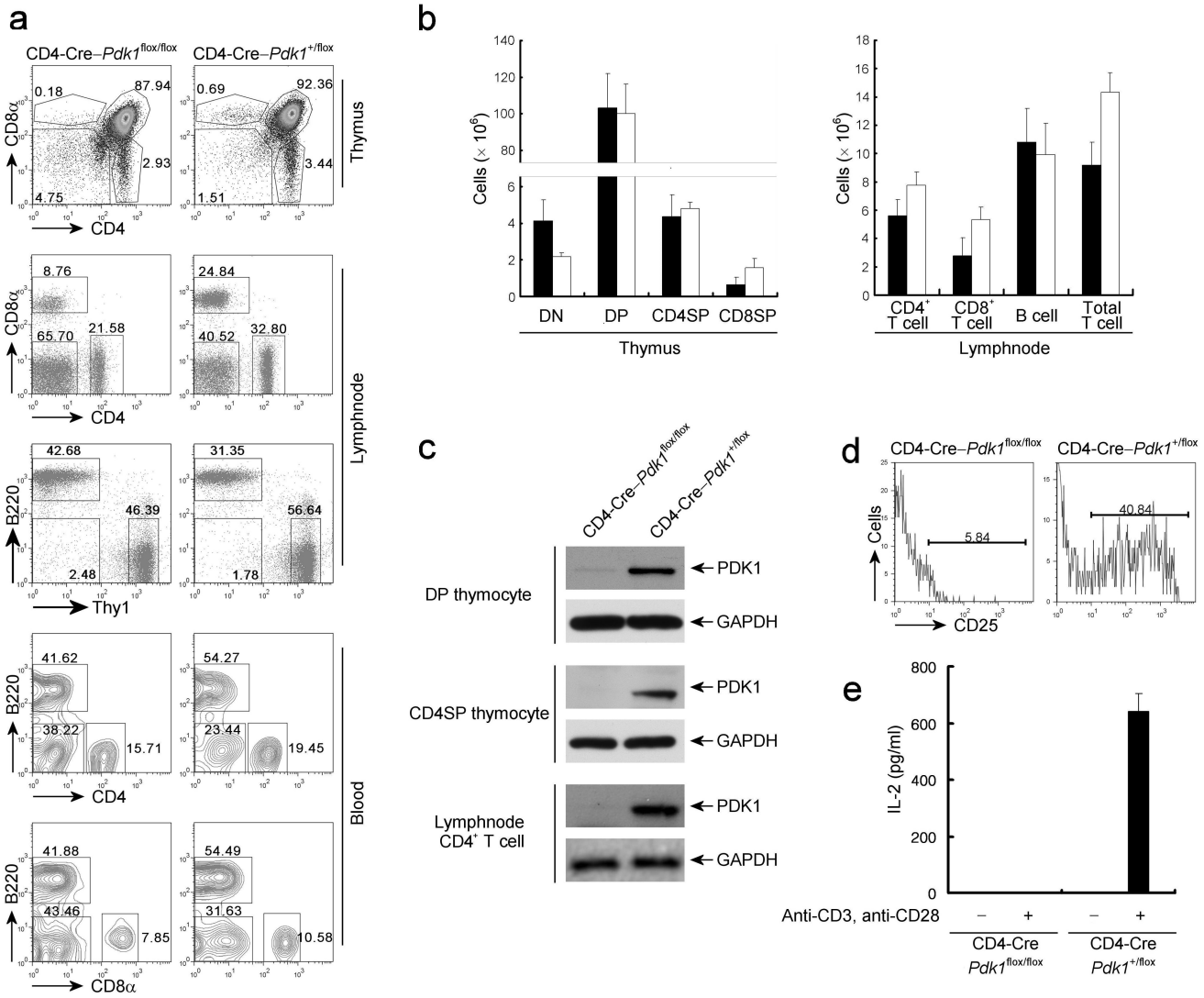

Figure 1. PDK1 affects activation but not survival of mature thymocytes

(**a,b**) Flow cytometric analyses of lymphocytes from thymus, blood, and peripheral lymph nodes (PLN) of CD4-Cre–*Pdk1*$^{flox/flox}$ and CD4-Cre–*Pdk1*$^{+/flox}$ mice (**a**). The number of each cell type in thymus and lymphnode is indicated (n = 4) (**b**). (**c**) PDK1 protein levels analyzed by immunoblot analysis of lysates from sorted double-positive thymocytes, CD4 single-positive (CD4SP) thymocytes, and peripheral lymph node CD4$^+$ T cells from CD4-Cre–*Pdk1*$^{flox/flox}$ (filled bars) and CD4-Cre–*Pdk1*$^{+/flox}$ (open bars) mice. (**d,e**) Flow cytometry for cell surface expression of CD25 on (**d**) and ELISA assay for soluble IL-2 production from (**e**) sorted CD4 SP thymocytes from CD4-Cre–*Pdk1*$^{flox/flox}$ and CD4-Cre–*Pdk1*$^{+/flox}$ mice stimulated with anti-CD3–anti-CD28 for 24 h. Data are representative of at least three independent experiments (error bars (**e**), s.d. of triplicate samples).

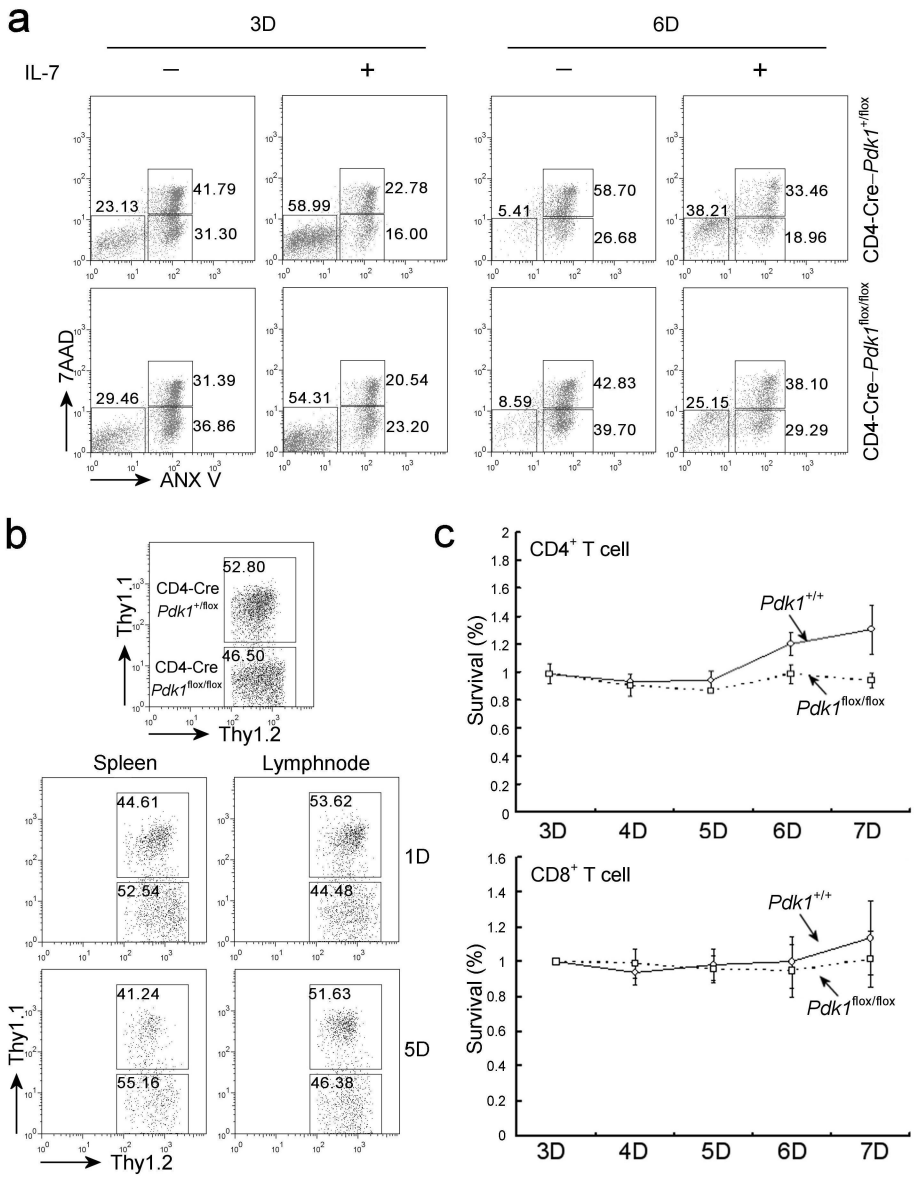

Figure 2. PDK1 is not required for CD4$^+$ T cell survival

(**a**) Flow cytometric analyses of thymocytes from CD4-Cre–*Pdk1*$^{flox/flox}$ and CD4-Cre–*Pdk1*$^{+/flox}$ mice after culturing for 3 and 6 d in the presence (+) or absence (−) of exogenous IL-7, followed by staining with annexin V–7-amino-actinomycin D (7AAD) and gating on CD4$^+$CD8$^-$ cells. Numbers indicate percent 7AAD and annexin V–double positive cells (top), annexin V–positive cells (right bottom) or 7AAD–annexin V–negative cells (left bottom). (**b**) Flow cytometry of CD4$^+$ T cells isolated from lymph nodes of recipient Thy1.1+ mice that received a 1:1 mix of CD4-Cre–*Pdk1*$^{flox/flox}$ Thy1.2$^+$ and CD4-Cre–*Pdk1*$^{+/flox}$ Thy1.2$^+$ CD4$^+$ T cells. At day 1 and day 5 after transfer the ratio of transferred cells (Thy1.2$^+$) were analyzed. Top panel shows the mixture of cells before transfer. (**c**) Flow cytometry of total splenocytes (*Pdk1*$^{flox/flox}$ or *Pdk1*$^{+/+}$) infected with retrovirus and 3 d later the ratio of GFP$^+$ to GFP$^-$ CD4$^+$ T cell (top panel) or CD8$^+$ T cell (bottom panel)

was set to 1; each following day the ratios were evaluated. Data are representative of two (**a,b**) or three (**c**) independent experiments (error bars (**c**), s.d. of triplicate samples).

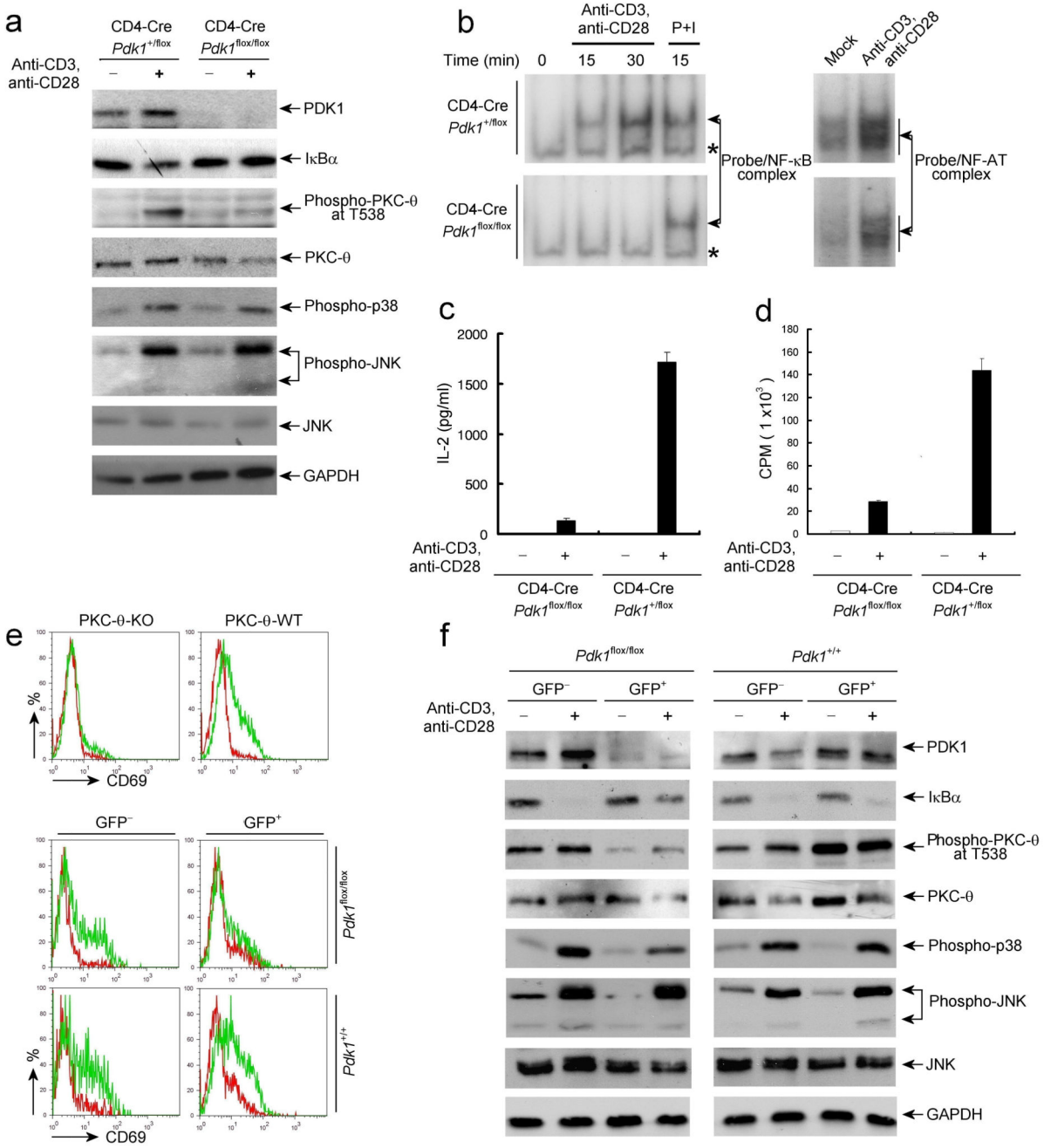

Figure 3. PDK1 deletion specifically affects NF-κB activation in both primary and effector CD4$^+$ T cells

(**a**) Immunoblot for phosphorylation of PKC-θ, JNK, and p38 MAPK and IκBα degradation in CD4-Cre–*Pdk1*$^{flox/flox}$ and CD4-Cre–*Pdk1*$^{+/flox}$ lymph node CD4$^+$ T cells stimulated with anti-CD3–anti-CD28 for 20 min. (**b**) EMSA for NF-κB and NF-AT DNA binding in nuclear extracts of CD4-Cre–*Pdk1*$^{flox/flox}$ and CD4-Cre–*Pdk1*$^{+/flox}$ lymph node CD4$^+$ T cells stimulated with anti-CD3–anti-CD28 for 15 and 60 min (for NF-κB) or 12 h (for NF-AT). PMA plus ionomycin (P+I) was used for control. Asterisk indicates nonspecific signals. (**c**)

ELISA for IL-2 production from primary CD4-Cre–*Pdk1*$^{flox/flox}$ and CD4-Cre–*Pdk1*$^{+/flox}$ CD4^{+} T cells stimulated with anti-CD3–anti-CD28 for 24 h. (**d**) Proliferation of CD4-Cre–*Pdk1*$^{flox/flox}$ and CD4-Cre–*Pdk1*$^{+/flox}$ CD4^{+} T cells stimulated with anti-CD3–anti-CD28 for 36 h and then incubated with 1 μCi of [^{3}H]-thymidine for 12 h. (**e**) Flow cytometry for CD69 on wild-type, PKC-θ-KO (*Pkcq*$^{-/-}$) CD4^{+} T cells, and *Pdk1*$^{flox/flox}$ or *Pdk1*$^{+/+}$ CD4^{+} T cells transduced with MSCV retrovirus encoding Cre and EGFP. Cells were stimulated with anti-CD3–anti-CD28 (green line) or left unstimulated (red line) for 8 h and then gated by CD4^{+} and by GFP^{+} and GFP^{-}. (**f**) Immunoblot for phosphorylation of PKC-θ, JNK, and p38 MAPK and IκBα degradation in *Pdk1*$^{flox/flox}$ or *Pdk1*$^{+/+}$ effector CD4^{+} T cells transduced with Cre-EGFP-expressing retrovirus and then stimulated with anti-CD3–anti-CD28 for 20 min. Data are representative of three (**a,b,c,e,f**) or four (**d**) independent experiments (error bars (**c,d**), s.d. of triplicate samples).

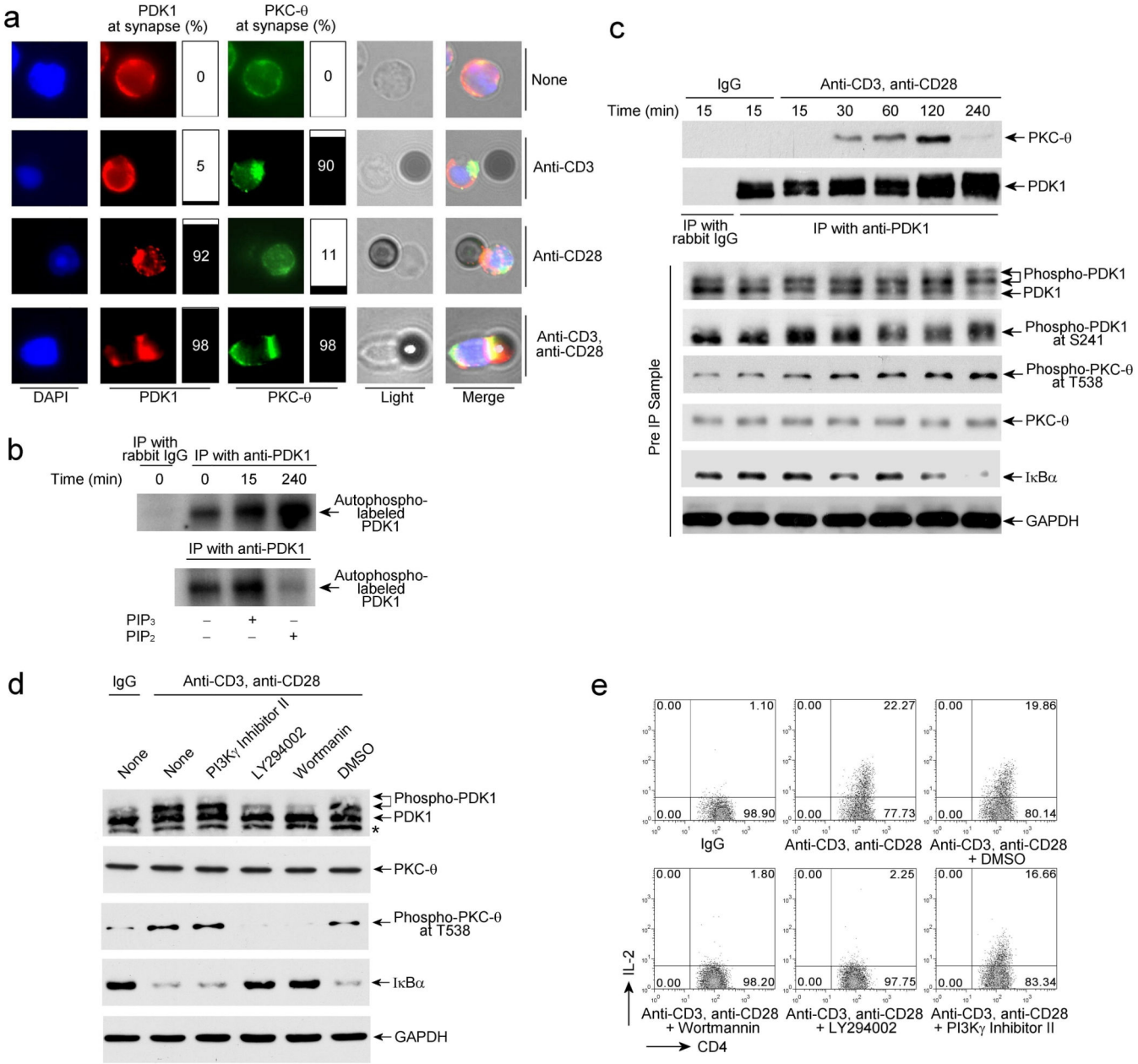

Figure 4. PDK1 is recruited by CD28 ligation and integrates CD28 signaling and T cell receptor signaling to activate NF-κB through phosphorylation-dependent binding to PKC-θ

(**a**) Immunofluorescent microscopy for localization of PDK1 and PKC-θ in primary mouse $CD4^+$ T cells incubated with dynabeads (M450) coated with IgG, anti-CD3, anti-CD28, or anti-CD3–anti-CD28. The nucleus was stained with DAPI. (**b**) Autophosphorylation activity of PDK1 isolated from Jurkat T cell analyzed by *in vitro* kinase assay following stimulation with anti-CD3–anti-CD28 or upon addition of lipid containing either phosphatidyl choline (PS)-phosphatidyl serine (PS)-PIP_2 or PC-PS-PIP_3. (**c**) Immunoblot analysis of binding between PDK1 and PKC-θ, phosphorylation of PDK1 and PKC-θ, and degradation of IκBα in Jurkat T cell lysates stimulated with anti-CD3–anti-CD28 for the indicated times. (**d**)

Immunoblot for phosphorylation of PDK1 and PKC-θ and degradation of IκBα in Jurkat T cells stimulated the PI3K inhibitor PI3Kγ-inhibitorII, LY294002, or wortmannin. (**e**) Flow cytometry for intracellular IL-2 production in primary $CD4^+$ T cell following treatment with the PI3K inhibitors (PI3Kγ-inhibitorII, LY294002, or wortmannin). Data are representative of two (**a,b**), three (**d,e**) or five (**c**) independent experiments.

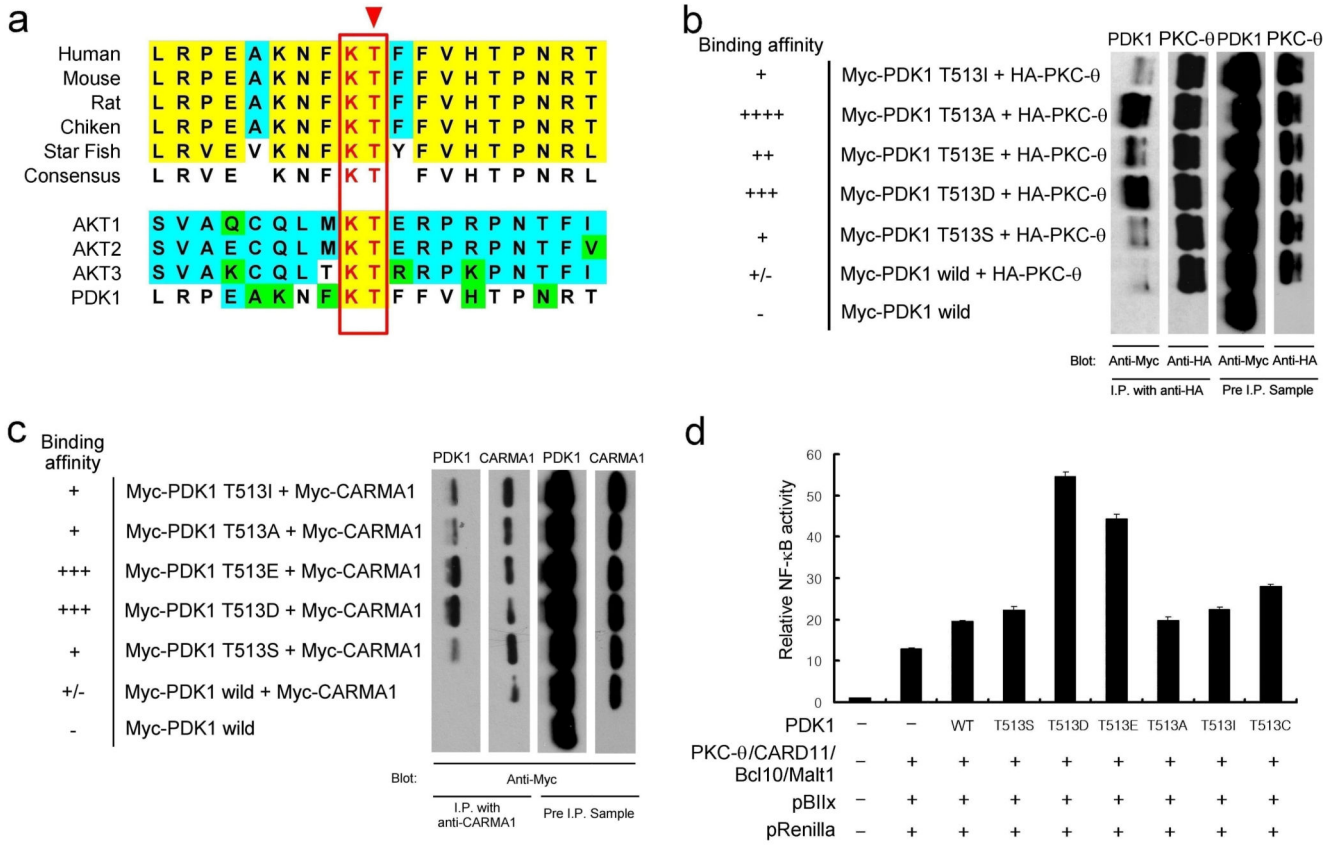

Figure 5. Phosphorylation of a conserved residue on PDK1 plays a central role in PKC-θ–CARMA1–Bcl10–MALT1-mediated NF-κB activation

(**a**) Primary amino acid sequence of human PDK1 near threonine 513 was aligned with those of other species of PDK1 and AKT family members (AKT1, AKT2, and AKT3). (**b,c**) Binding between PDK1 bearing mutations at T513 and PKC-θ (**b**) or CARMA1 (**c**) was analyzed by immunoprecipitation and immunoblot analysis in transfected HEK293 cell lysates. (**d**) Effect of PDK1 mutation at T513 on PKC-θ–CARMA1–Bcl10–MALT1-mediated NF-κB activation was analyzed by NF-κB luciferase reporter assay in HEK293 cells. Data are one representative of three (**d**) or five (**b,c**) independent experiments (error bars (**d**), s.d. of triplicate samples).

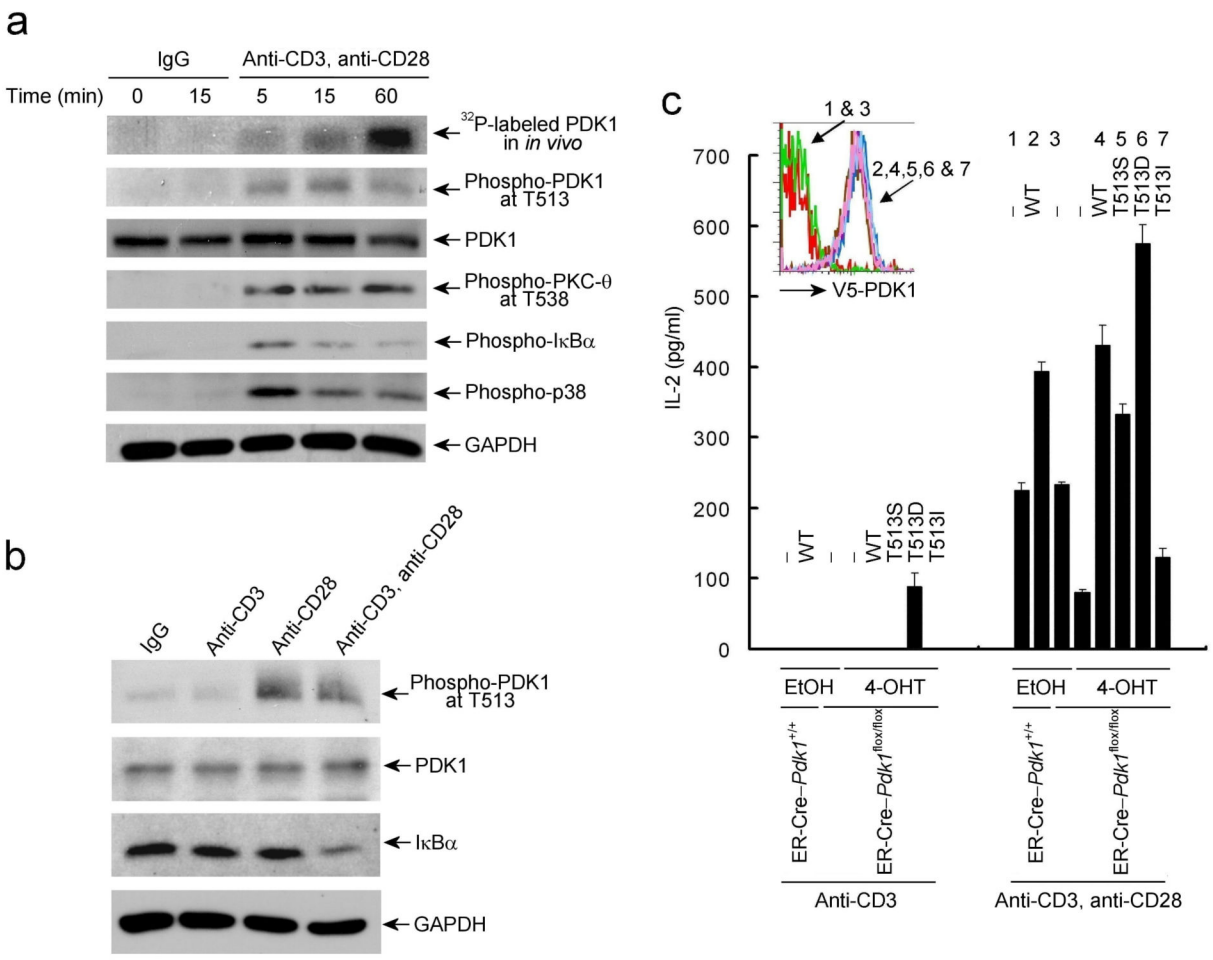

Figure 6. Phosphorylation of T513 on PDK1 plays a central role in CD4$^+$ T cell activation

(**a**) Phosphorylation of PDK1 in primary CD4$^+$ T cells in response to anti-CD3–anti-CD28 stimulation was analyzed by both *in vivo* metabolic labeling with ^{32}P-inorganic phosphate and immunoblot analysis with anti-phospho PDK1 specific to T513 phosphorylation. (**b**) Immunoblot analysis of phosphorylation of PDK1 T513 in primary CD4$^+$ T cell following anti-CD3, anti-CD28 or anti-CD3–anti-CD28 stimulation for 30 min with anti-phospho PDK1. (**c**) ELISA for IL-2 production in PDK1-deleted CD4$^+$ T cells transduced with PDK1 T513-expressing lentiviruses. Complemented PDK1 protein expression was confirmed by intracellular flow cytometry with V5 antibody. Data are representative of two (**a,b**) or three (**c**) independent experiments (error bars (**c**), s.d. of triplicate samples).