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Transition from Heterotypic to Homotypic PDK1 Homodimerization Is Essential for TCR-Mediated NF-κB Activation

Jung-Ah Kang,*† Sang Phil Jeong,*† Daeho Park,* Matthew S. Hayden,‡§ Sankar Ghosh,‡ and Sung-Gyoo Park*,†

Strong NF-κB activation requires ligation of both the CD28 coreceptor and TCR. Phosphoinositide-dependent kinase 1 (PDK1) acts as a scaffold by binding both protein kinase C0 (PKC0) and CARMA1, and is therefore essential for signaling to NF-κB. In this article, we demonstrate the importance of PDK1 Thr513 phosphorylation in regulating the intermolecular organization of PDK1 homodimers. Thr513 is directly involved in heterotypic PDK1 homodimer formation, in which binding is mediated through the pleckstrin homology (PH) and kinase domains. Upon activation, phosphorylated Thr513 instead mediates homotypic intermolecular binding through the PH domains. Consequently, cell-permeable peptides with a Thr513 to Ile derivative (protein transduction domain [PTD]-PDK1-Thr513-Ile) bound the kinase domain, whereas a Thr513-to-Asp peptide (PTD-PDK1-Thr513-Asp) bound the PH domain. PTD-PDK1-Thr513-Ile blocked binding between PDK1 and PKC0, phosphorylation of PKC0 Thr538, and activation of both NF-κB and AKT. In contrast, PTD-PDK1-Thr513-Asp selectively inhibited binding between PDK1 and CARMA1, and blocked TCR/CD28-induced NF-κB activation. Therefore, Thr513 phosphorylation regulates a critical intermolecular switch governing PDK1 homodimer structure and the capacity to interact with downstream signaling pathway components. Given the pleiotropic functions of PDK1, these data may open the door to the development of immunosuppressive therapies that selectively target the PDK1 to NF-κB pathway in T cell activation. The Journal of Immunology, 2013, 190: 000–000.

Phosphoinositide 3-kinases (PI3Ks) are a family of unique, conserved, intracellular lipid kinases that phosphorylate the 3'-hydroxy group of phosphatidylinositol or phosphoinositides (1). This phosphorylation induces the activation of many intracellular signaling pathways, and thereby mediates cellular functions, such as metabolism, survival, and polarity (2). Activation of the various cell-surface receptors induces the activation of PI3K, and this activated PI3K converts the membrane phospholipid phosphatidylinositol-4,5-bisphosphate into phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) (2). During this process, phosphoinositide-dependent kinase 1 (PDK1) is activated by PtdIns(3,4,5)P3, and this activated PDK1 mediates multiple signaling pathways that are important for cell metabolism, survival, and activation (3). Further, PDK1 may act as a “master regulator” of the protein kinase A/protein kinase G/protein kinase C (PKC) family of kinases (4). The most well-characterized target of PDK1 is AKT; in fact, PDK1 was initially identified as an AKT kinase (4, 5). AKT activity is regulated via Thr308 phosphorylation by PDK1 (5, 6) and Ser473 phosphorylation by mammalian target of rapamycin (7, 8).

PDK1 has important roles in activation and function of immune cells (4, 9–13). Previous studies have established that, during coordinated CD28/TCR stimulation of T cells, PDK1 is essential for efficient activation of PKC0 by the PI3K pathway (4) and subsequent assembly of the CARMA1-BCL10-MALT1 (CBM) complex to activate NF-κB (14, 15). PDK1 also plays a crucial role in T cell development, because conditional deletion of PDK1 in double-negative (DN) thymocytes blocks T cell development at the DN4 stage by impairing pre-TCR–induced proliferation (16). However, deleting PDK1 in double-positive (DP) thymocytes does not affect CD4+ single-positive thymocyte development, whereas CD8 single-positive thymocyte development is largely affected by PDK1 deletion (4).

PDK1 is composed of a kinase and pleckstrin homology (PH) domain. The kinase domain phosphorylates substrate protein, and the PH domain binds PtdIns(3,4,5)P3 during PDK1-mediated substrate activation (4). Phosphorylation of PDK1 Thr513 is important for binding to PKC0 and is induced by CD28/TCR stimulation during T cell activation (4). Initially, the phosphorylation site was found to be autophosphorylated upon binding to the phosphor–lipid complex (17). Previous studies have suggested PDK1 is initially homodimerized, preventing substrate binding (17, 18). However, mutation of PDK1 Thr513 to Glu (phosphomimic) destabilizes homodimerization and increases binding between PDK1 and AKT, which results in AKT activation through Thr308 phosphorylation (18).
In this report, we found that PDK1 phosphorylation at Thr\(^{513}\) induces changes in interdomain binding, thus regulating binding between PDK1 and PKC\(\alpha\) or CARMA1. The phosphorylation destabilized the binding kinase domain and PH domain, and increased the binding between PH domains. Moreover, nonphosphomimic Thr\(^{513}\) peptide binds the PDK1 kinase domain and inhibits binding of PDK1 to PKC\(\alpha\). In addition, phosphomimic Thr\(^{513}\) peptide binds to the PDK1 PH domain and inhibits binding of PDK1 to CARMA1 and binding between PH domains, which results in impaired CD28/TCR stimulation and T cell activation. Given the pleiotropic functions of PDK1, these data may open the door to the development of immunosuppressive therapies that selectively target the PDK1 to NF-κB pathway in T cell activation.

Materials and Methods

Cell culture, Abs, and peptides

HEK293 cells were maintained in DMEM supplemented with 5% FBS. Jurkat T cells were maintained in RPMI 1640 supplemented with 5% FBS. Anti-IκBα and anti-GAPDH Abs were purchased from Santa Cruz. Anti-Myc Ab was purchased from Cell Signaling. Anti-CARM1 Ab was purchased from Chemicon. Anti-HA Ab was purchased from Sigma. Phospho-Myc Ab was purchased from Cell Signaling. Anti-CARMA1 Ab was obtained from Stem Cell Research. Purified CD4\(^{+}\) T cells were activated with anti-CD3, anti-CD28, anti-human CD3, and anti-mouse CD25 Abs were purchased from eBioscience. Peptides, in vivo mouse CD4, PE-conjugated anti-mouse CD69, and PerCP Cy5.5-conjugated anti-mouse CD3, anti-mouse CD28, and anti-mouse CD25 were purchased from BD Biosciences.

Plasmids

pCDNA-Myc-PDK1 and pCDNA-HA-PDK1 were constructed by insertion of Myc sequence tagged-pPdk1 open reading frame (ORF) or Ha sequence tagged- Pdk1 ORF into the pCDNA3. pCDNA-Myc-PDK1-PH and pCDNA-HA-PDK1-PH were constructed by insertion of Myc sequence tagged-Pdk1 PH domain sequences or Ha sequence tagged-Pdk1 PH domain sequences into pCDNA3. A QuikChange II XL site-directed mutagenesis kit (Stratagene) was used for mutagenesis of pCDNA-Myc-PDK1, pCDNA-HA-PDK1, pCDNA3-Myc-PDK1-PH, and pCDNA3-HA-PDK1-PH. pEGZ-HA-PDK1-PH was constructed by insertion of the Pkg \(\beta\) gene luciferase assay

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For fluorescence microscopy, primary CD4\(^{+}\) T cells were incubated with FITC-peptide for 30 min at 37°C. After incubation, T cells were plated on poly-L-lysine-coated coverslips for 15 min at 37°C, then fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were incubated with DAPI for nuclear staining. Results were analyzed by Zeiss confocal microscopy.

Results

Binding of the PDK1 PH to kinase domains is destabilized by Thr\(^{513}\) phosphorylation

PDK1 Thr\(^{513}\) phosphorylation is essential for TCR-CD28 stimulation-mediated NF-κB activation (4). This phosphorylation was increased...
by TCR-CD28 stimulation (Fig. 1A) (4). Many proteins, including PDK1, possess a PH domain. The AKT also has a PH domain, and it has been shown that the phosphorylation site is important for binding of PDK1 to PKCθ. Our binding analysis showed that overexpressed PDK1 PH domain (401–550) bound to the PDK1 kinase domain (1–341) in HEK293 cells (Fig. 1B). Mutation of Thr513 to Asp (phosphomimic) decreased binding between the PH and kinase domains, but Ile (phosphorylation-disabled) increased binding (Fig. 1B). The PDK1 region responsible for PKCθ binding was analyzed in the ex vivo system by overexpressing each PDK1 deletion fragment in HEK293 cells. The data indicated that PDK1 kinase domain is required for binding to PKCθ, and the region overlaps with the PH domain–binding region (Supplemental Fig. 1). Thus, full-length PDK1 Thr513-Asp binds to PKCθ more strongly than to PDK1 wild-type (WT) because phosphorylation destabilizes binding between the kinase and PH domains.

**Phosphomimic at PDK1 Thr513 induced strong homotypic binding of PDK1 PH domains**

PDK1 is dimerized, and Thr513 on PDK1 is autophosphorylated via transphosphorylation (17). A recent study showed that a Thr513 phosphorylation mimic destabilizes homodimerization of PDK1, thus enabling PDK1-substrate binding (18). The previous study used fluorescence resonance energy transfer methods. We used coimmunoprecipitation and immunoblot analyses with tagged PDK1-expressing HEK293 cells. Our data showed that Myc-tagged WT PDK1 coimmunoprecipitated with HA-tagged WT PDK1; in contrast, Myc-tagged WT PDK1 did not coimmunoprecipitate with anti-HA Ab in the absence of HA-tagged WT PDK1 in HEK293 cells (Fig. 2A). Initially, we expected the Thr513-Asp mutation to abolish binding between tagged PDK1 mutant derivatives. However, Myc-tagged PDK1 Thr513-Asp still coim-
munoprecipitated with HA-tagged PDK1 Thr<sup>513</sup>-Asp (Fig. 2A). Because binding of PDK1 PH to kinase domains is destabilized by Thr<sup>513</sup> phosphorylation and PDK1 kinase domain is the substrate-binding site, Thr<sup>513</sup> phosphorylation may lead to binding between PDK1 PH domains. Thus, we tested whether the PDK1 PH domain dimerizes by using differentially tagged PDK1 PH domains expressed in HEK293 cells. Fig. 2B shows that Myc-tagged-PDK1 PH domain communoprecipitated with HA-tagged PDK1 PH domain. In addition, Thr<sup>512</sup>-Asp mutation of the PDK1 PH domain dramatically increased binding between tagged domains.

**Phosphomimic PDK1 Thr<sup>513</sup> region-containing peptide binds to PDK1 PH domain and unphosphomimic PDK1 Thr<sup>513</sup> peptide binds to PDK1 kinase domain**

Thr<sup>513</sup> phosphorylation is important for T cell activation because it increases binding between PDK1 and PKC<sub>u</sub>, which mediates CBM complex-mediated NF-κB activation (4). In addition, it has been found that Thr<sup>513</sup> is phosphorylated by transautophosphorylation (17). Sequence comparison of the Thr<sup>513</sup> region with the phosphorylation sites of PDK1 substrates showed the Thr<sup>513</sup> region is similar to the PDK1 phosphorylation motif (Fig. 3A). To test whether the Thr<sup>513</sup> phosphorylation site can directly regulate binding between PDK1 and PKC<sub>u</sub> and binding between PH domains, we designed peptides containing the Thr<sup>513</sup> site and PTD. PTD-PDK1-Thr<sup>515</sup>-Ile is composed of 17 PTD amino acids and 6 amino acids before and after the PDK1 Thr<sup>513</sup>-Ile substitution; PTD-PDK1-Thr<sup>515</sup>-Asp is composed of 17 PTD amino acids and 6 amino acids before and after the PDK1 Thr<sup>513</sup>-Asp substitution (Fig. 3B).

With the peptides, we tested binding between the peptides and the PDK1 kinase and PH domains. Our pull-down assay showed the PDK1 kinase domain bound to PTD-PDK1-Thr<sup>513</sup>-Ile peptide-
conjugated M450 beads and the PDK1 PH domain bound to PTD-PDK1-Thr513-Asp peptide-conjugated M450 beads (Fig. 3C). In addition, PTD-PDK1-Thr513-Ile and PTD-PDK1-Thr513-Asp peptide-conjugated M450 beads bound to PDK1 from activated mouse primary CD4+ T cells. These data indicated that the peptide was accessible to the binding region on PDK1 only after T cell activation. In these experiments, the more PDK1 bound to PTD-PDK1-Thr513-Ile than PTD-PDK1-Thr513-Asp peptide-conjugated M450 beads (Fig. 3D).

**PDK1 Thr513 region–containing peptides regulate binding of PDK1 to PKC**

We used coimmunoprecipitation and immunoblot analyses to test whether the designed peptides regulate binding between PDK1 and PKC. We found that PTD-PDK1-Thr 513-Ile inhibited binding between overexpressed PDK1 and PKC0 in HEK293 cells (Fig. 4A). Binding between PDK1 PH domains was inhibited by PTD-PDK1-Thr513-Asp (Fig. 4B). Moreover, PTD-PDK1-Thr513-Ile inhibited binding between PDK1 and PKC0 in Jurkat T cells stimulated with anti-CD3 and anti-CD28 Abs (Fig. 4C). However, PTD-PDK1-Thr513-Asp did not affect binding between PDK1 and PKC0, but did inhibit binding between PDK1 and CARMA1 (Fig. 4C). In Jurkat T cells, PTD-PDK1-Thr513-Ile inhibited binding between PDK1 Thr513-Ile proteins (Fig. 4D), and PTD-PDK1-Thr513-Asp inhibited binding between PDK1 Thr513-Asp proteins with or without stimulation by anti-CD3 and anti-CD28 Abs (Fig. 4E). We also tested the effect of peptides on binding between WT PDK1 proteins. PTD-PDK1-Thr513-Ile strongly affected binding between WT PDK1 proteins when Jurkat T cells were not stimulated (Fig. 4F). However, PTD-PDK1-Thr513-Asp strongly affected binding between WT PDK1 proteins in Jurkat T cells stimulated with anti-CD3 and anti-CD28 Abs (Fig. 4F).

**TCR-CD28–mediated NF-κB activation is inhibited by PDK1-PH Thr513-Asp and PDK1-PH Thr513-Ile**

Thr513 phosphorylation in the PDK1 PH domain is important for binding between the PDK1 kinase domain and PKC0 and between PH domains. The PDK1 PH Thr513-Ile mutant domain binds to the kinase domain, and the PDK1 PH Thr513-Asp mutant domain binds to the PH domain. Thus, PDK1 PH Thr513-Ile may inhibit binding of PDK1 to PKC0, and PDK1 PH Thr513-Asp may inhibit homodimerization of PH domains during T cell activation. Previous reports have shown that PKC0-mediated CBM complex formation is required for NF-κB activation (20–22). Overexpression of these proteins in HEK293 cells induced strong NF-κB activation, which was enhanced further by PDK1 overexpression. In this system, overexpression of the PDK1 PH domain inhibited NF-κB activation. In addition, overexpression of the PDK1 PH Thr513-Ile domain or PDK1 PH Thr513-Asp domain inhibited NF-κB activation (Fig. 5A). The data indicate that binding of PKC0 to PDK1 and PDK1 homotypic dimerization is important for PKC0 and CBM-mediated NF-κB activation. However, these data were obtained from an artificial NF-κB activation system in HEK293 cells, so we determined whether NF-κB activation can be inhibited by PDK1 PH Thr513-Ile or PDK1 PH Thr513-Asp expression in mouse primary CD4+ T cells (B) or Jurkat T cells (C) was determined by NF-κB reporter assay. The Renilla luciferase activity was used as a reference for normalization of gene expression in transiently transfected cells. Results are presented as mean ± SD. Student t test: *p < 0.05, **p < 0.01.

**FIGURE 5.** PDK1 PH domain inhibits PDK1-mediated NF-κB activation in T cells. (A) The effect of PDK1 PH domain expression on PDK1-PKC0-CBM-mediated NF-κB activation was determined by NF-κB-reporter assay. (B and C) The effect of PDK1 PH domain expression on TCR-CD28–mediated NF-κB activation in mouse primary CD4+ T cells (B) or Jurkat T cells (C) was determined by NF-κB reporter assay. The Renilla luciferase activity was used as a reference for normalization of gene expression in transiently transfected cells. Results are presented as mean ± SD. Student t test: *p < 0.05, **p < 0.01.

PDK1 PH WT, PDK1 PH Thr513-Ile, or PDK1 PH Thr513-Asp expression plasmids significantly reduced TCR-CD28–mediated NF-κB activation in mouse primary CD4+ T cells (Fig. 5B) and Jurkat T cells (Fig. 5C).
PDK1 Thr513 region–containing peptides inhibited NF-κB activation in activated T cells, reducing IL-2 production

Interestingly, PTD-PDK1-Thr513-Ile inhibited PKC0 phosphorylation and IkBα degradation in activated CD4+ T cells, but PTD-PDK1-Thr513-Asp inhibited IkBα degradation and not PKC0 phosphorylation (Fig. 6A). In addition, PTD-PDK1-Thr513-Ile inhibited AKT Thr108 phosphorylation, but PTD-PDK1-Thr513-Asp did not (Supplemental Fig. 2). Thus, PTD-PDK1-Thr513-Asp does not affect the phosphorylation of downstream molecules even though the peptide affects the NF-κB activation during the CD4+ T cell activation. In addition to our investigation on the short-term temporal NF-κB activation through the detection of IkBα degradation, long-term total NF-κB activation levels in mouse primary CD4+ T cells during TCR-CD28 stimulation were determined using NF-κB activation reporter plasmids. PTD-PDK1-Thr513-Ile significantly inhibited NF-κB activation in CD4+ T cells stimulated with anti-CD3 and anti-CD28 Abs. PTD-PDK1-Thr513-Asp also inhibited NF-κB activation, but the inhibitory effect was less substantial (Fig. 6B). In addition, expression of IL-2, the most important NF-κB target during T cell activation, was also inhibited by PTD-PDK1-Thr513-Ile and PTD-PDK1-Thr513-Asp (Fig. 6C). The peptides also reduced the levels of secreted IL-2 protein from activated CD4+ T cells (Fig. 6D). The data indicate that blocking of PDK1 binding to PKC0 by PTD-PDK1-Thr513-Ile and disruption of PDK1 homotypic dimerization by PTD-PDK1-Thr513-Asp inhibits TCR-CD28–mediated NF-κB activation (Fig. 6E).

**Peptides containing the PDK1 Thr513 region inhibited TCR-mediated NF-κB activation**

To test whether the PTD-tagged peptide is taken up by T cells, we conjugated PTD-PDK1-Thr513-Asp and PTD-PDK1-Thr513-Ile with FITC and analyzed by flow cytometry. The FITC-conjugated peptides were transduced to almost all isolated primary CD4+ T cells (Fig. 7A). Fluorescence microscopy confirmed the transduced peptides lay within the CD4+ T cells (Fig. 7B).
Our data showed that PTD-PDK1-Thr<sup>513</sup>-Ile and PTD-PDK1-Thr<sup>513</sup>-Asp inhibited TCR-CD28-mediated NF-κB activation by inhibiting binding between PDK1 and PKC<sub>u</sub> and between PDK1 and CARMA1. In addition, PDK1 is linked to T cell activation because genetic deletion of Pdk1 in CD4<sup>+</sup> T cells results in defective NF-κB activation during TCR-CD28-mediated T cell activation (4). Thus, we tested whether our peptides could inhibit CD4<sup>+</sup> T cell activation in primary mouse CD4<sup>+</sup> T cells activated with anti-CD3 and anti-CD28. PTD-PDK1-Thr<sup>513</sup>-Ile and PTD-PDK1-Thr<sup>513</sup>-Asp inhibited surface expression of CD69 (Fig. 7C, Supplemental Fig. 3) and CD25 (Fig. 7D, Supplemental Fig. 3), and control PTD peptide did not affect expression of activation surface markers. In addition, PTD-PDK1-Thr<sup>513</sup>-Ile more strongly inhibited CD4<sup>+</sup> T cell activation than did PTD-PDK1-Thr<sup>513</sup>-Asp (Fig. 7C, 7D), like data shown in Fig. 6.

**Discussion**

TCR-CD28-mediated NF-κB activation is important for CD4<sup>+</sup> T cell activation because NF-κB regulates the expression of cytokine genes and the survival of activated T cells (4). Our previous study showed that PDK1 is essential for NF-κB activation, and Thr<sup>513</sup> phosphorylation is important for the regulation of the binding between PDK1 and its substrates, PKC<sub>u</sub> and CARMA1. However, we did not elucidate the detailed mechanism by which the phosphorylation regulates the binding. Recently, Thr<sup>513</sup> phosphorylation has been suggested to be important for the regulation of PDK1 homodimerization. Fluorescence resonance energy transfer data demonstrated that PDK1 homodimerizes, and that this homodimerization inhibited PDK1-substrate binding (18). In addition, the PDK1 homodimer is destabilized by phosphomimic mutation at Thr<sup>513</sup>, enabling binding between PDK1 and its substrate (17, 18). Our coimmunoprecipitation-mediated binding analysis showed that PDK1 kinase domain binds to PDK1 PH domain during PDK1 homodimerization. In addition, phosphomimic mutation at Thr<sup>513</sup> destabilized binding between the PDK1 kinase and PH domains. Because PDK1 does not form an intramolecular bond between the N- and C-terminal domains (18), we hypothesized that homodimerization occurs via intermolecular binding between the PDK1 kinase and PH domains. We previously demonstrated that a phosphomimic of PDK1 Thr<sup>513</sup> increases binding between PDK1 and PKC<sub>u</sub>. Thus, we thought that destabilization of PDK1 homodimer formation through phosphomimic at Thr<sup>513</sup> is important for PDK1-mediated NF-κB activation during T cell activation. Interestingly, however, Thr<sup>513</sup> phosphomimic still formed a homodimer, but with increased homotypic binding between PH domains.

PDK1 Thr<sup>513</sup> is a transautophosphorylation site (17); thus, Thr<sup>513</sup> is a substrate for PDK1 kinase. Sequence alignment of the Thr<sup>513</sup> region of PDK1 with PDK1 substrate sequences indicates the regions are similar to the substrate motif. In addition, PTD-PDK1-Thr<sup>513</sup>-Ile bound to PDK1 kinase domain, but the phosphomimic form, PTD-PDK1-Thr<sup>513</sup>-Asp, did not. Instead, PTD-PDK1-Thr<sup>513</sup>-Asp bound the PH domain, whereas PTD-PDK1-Thr<sup>513</sup>-Ile did not. Thus, the model developed in our previous study can be modified. Initially, PDK1 dimerizes via binding between the PDK1 kinase and PH domains, but phosphorylation of PDK1 at Thr<sup>513</sup> induces binding between PDK1 PH domains, thus exposing the kinase domain to PKC<sub>u</sub>. In addition, PDK1 PH domain homodimerization creates a docking site for CARMA1; the resulting PDK1/PKC<sub>u</sub>/CARMA1 complex mediates NF-κB activation during TCR-CD28-mediated T cell activation.

PTD-PDK1-Thr<sup>513</sup>-Asp did not inhibit binding of PDK1 to PKC<sub>u</sub> or PKC<sub>u</sub> phosphorylation, but did inhibit TCR-CD28-mediated NF-κB activation. This inhibition might be caused by...
inhibition of homotypic binding between PDK1 PH domains, dramatically reducing binding of PDK1 to CARMA1. These data imply that binding to partners such as CARMA1 requires PDK1 PH domain-mediated dimerization, whereas AKT phosphorylation and PKCθ phosphorylation do not require PH domain-mediated homotypic binding. In addition, we found that blocking of PDK1 PH domain-mediated dimerization does not affect the AKT activation, and AKT overexpression did not affect the PDK1 Thr^{513}-Asp dimerization. These data may indicate that PH domain binding-mediated PDK1 dimer formation occurred in cells even though this dimer formation is not essential for AKT activation. Thus, in T cell activation, PKCθ phosphorylation itself is not an indicator of NF-κB activation because PDK1-mediated signaling complex formation is required for activation. Therefore, PTD-PDK1-Thr^{513}-Asp can specifically regulate functions of PDK1 in TCR-CD28-mediated NF-κB activation through regulation of binding to CARMA1.

In summary, this report describes a detailed mechanism for PDK1-mediated NF-κB activation in T cells in response to coengagement of TCR and CD28. Particularly, PDK1 homodimerization without phosphorylation at Thr^{513} inhibits binding of PDK1 to PKCθ, but Thr^{513} phosphorylation-dependent homodimerization is essential for binding of PDK1 to both PKCθ and CARMA1, required for NF-κB activation during TCR-CD28-mediated T cell activation. In addition, our data showed that PDK1 Thr^{513} is a potential target for development of a regulator of PDK1 function in T cell activation.

Disclosures
The authors have no financial conflicts of interest.

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