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Phosphatidylinositol 4-Phosphate 5-Kinase α Activation Critically Contributes to CD28-Dependent Signaling Responses

Michela Muscolini,* Cristina Camperio,* Cristina Capuano,† Silvana Caristi,* Enza Piccolella,* Ricciarda Galandrini,† and Loretta Tuosto*

CD28 is one of the most relevant costimulatory receptors that deliver both TCR-dependent and TCR-independent signals regulating a wide range of signaling pathways crucial for cytokine and chemokine gene expressions, T cell survival, and proliferation. Most of the CD28-dependent signaling functions are initiated by the recruitment and activation of class IA PI3Ks, which catalyze the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate, thus generating the docking sites for key signaling proteins. Hence, PIP2 is a crucial substrate in driving the PI3K downstream signaling pathways, and PIP2 turnover may be an essential regulatory step to ensure the activation of PI3K following CD28 engagement. Despite some data evidence that CD28 augments TCR-induced turnover of PIP2, its direct role in regulating PIP2 metabolism has never been assessed. In this study, we show that CD28 regulates PIP2 turnover by recruiting and activating phosphatidylinositol 4-phosphate 5-kinases α (PIP5Kα) in human primary CD4+ T lymphocytes. This event leads to the neosynthesis of PIP2 and to its consumption by CD28-activated PI3K. We also evidenced that PIP5Kα signaling is required for both CD28 unique signals regulating IL-8 gene expression as well as for CD28/TCR-induced Ca2+ mobilization, NF-AT nuclear translocation, and IL-2 gene transcription. Our findings elucidate a novel mechanism that involves PIPS5Kα as a key modulator of CD28 costimulatory signals. The Journal of Immunology, 2013, 190: 000–000.

CD28 costimulatory receptor is a crucial determinant of the outcome of T lymphocyte activation. The engagement of CD28 by its natural ligand, B7.1/CD80 or B7.2/CD86, expressed on the surface of professional APC, lowers TCR activation threshold, thus leading to the enhancement of early signaling events necessary for efficient cytokine production, cell cycle progression, survival, and regulation of T cell effector responses (1, 2). CD28 is also able to act as a unique signaling receptor and to deliver TCR-independent autonomous signals, which account for its critical role in the regulation of proinflammatory cytokine/chemokine production and T cell survival (3).

A key modulator of CD28 signaling functions is PI3K. The cytoplasmic tail of CD28 contains the YMMN motif that, once tyrosine phosphorylated, binds to the Src homology 2 domain of the p85 regulatory subunit of class IA PI3Ks, which in turn recruits the catalytic p110 subunit (α, β, or δ p110 isoforms) (4, 5). Class IA PI3Ks phosphorylate phosphatidylinositol 4,5-biphosphate (PIP2) on carbon atom 3, thus generating phosphatidylinositol 3,4,5-triphosphate (PIP3) (6). PIP3 lipids are important docking sites by recruiting the pleckstrin homology of important mediators of both CD28 autonomous and TCR costimulatory signals (7), including phosphoinositide-dependent kinase 1 (PDK1), Itk (8), phospholipase Cγ1 (PLCγ1) (9), and protein kinase B (PKB/Akt) (10).

PI3K-dependent PDK1-PKB/Akt pathway ensures T cell survival by regulating the expression of both anti- and proapoptotic genes (11, 12). PI3K-PDK1-PKB/Akt has also been involved in CD28 signals regulating the activation of the transcription factor NF-κB (13–16). Moreover, PI3K-dependent generation of PIP3 also favors the recruitment and activation of PLCγ1. PLCγ1 hydrolyzes PIP2 in diacylglycerol and inositol trisphosphate (IP3), two second messengers that cooperate in inducing the activation of NF-AT (17), AP-1 (18), and NF-κB transcription factors (19, 20), essential for the expression of pivotal genes, including IL-2 (21). From all these evidences emerge the relevance of PI3K-mediated phosphorylation of PIP2 to PIP3 in multiple CD28 functions and the crucial role of PI3K metabolism in coupling CD28 to PI3K-dependent signaling pathways.

During T cell activation, PIP2 concentrates at the immunological synapse (IS), where it is rapidly synthesized and hydrolyzed by PLCγ1 and PLCβ (22). The main biosynthetic pathway of PIP2 involves phosphorylation of phosphatidylinositol 4-monophosphate (P4IP) at the D5 position of the inositol ring by phosphatidylinositol 4-phosphate 5-kinases (PIPS5K) (23). Three PIPS5K isoforms (α, β, and γ) have been identified (24–26). Although the visualization of endogenous PIPS5K isoforms has been often difficult, several data obtained in different cell systems evidenced differential subcellular localizations of each isoform.

Abbreviations used in this article: [Ca2+]i, intracellular calcium; GAM, goat anti-mouse; HA, hemagglutinin; IP3, inositol trisphosphate; IS, immunological synapse; PDK1, phosphoinositide-dependent kinase 1; PIP4, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-biphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate; PIP5K, phosphatidylinositol 4-phosphate 5-kinases; PKB, protein kinase B; PLCγ1, phospholipase Cγ1; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; SEE, staphylococcal enterotoxin E; siRNA, small interfering RNA.
PIPKαα, for instance, is localized at the plasma membrane, where it guarantees the local availability of PIP2 (27–29). Although CD28 has been previously described to augment TCR-induced turnover of PIP2 (30), its direct role in regulating PIP2 has never been assessed. In this study, we show that CD28 stimulation by B7.1/CD80 or agonistic Abs induces the recruitment and activation of PIPKαα in human primary CD4+ T lymphocytes. This event leads to the neosynthesis of PIP2 that is consumed by CD28-activated PIM3. By either small interference RNA (siRNA)-driven cell silencing or overexpressing a kinase-dead mutant, we evidenced that PIPKαα activation is required for both CD28 autonomous signals regulating IL-8 gene expression as well as for CD28-TCR-induced Ca2+ mobilization, NF-AT nuclear translocation, and IL-2 gene transcription. Our findings identify PIPKαα as a critical mediator of CD28-dependent responses.

Materials and Methods

Cells, Abs, and reagents
Human primary CD4+ T cells were enriched from PBMCs by MACS microbead sorting (Miltenyi Biotec, Milan, Italy) and cultured in RPMI 1640 supplemented with 5% human serum (Euroclone, UK), 1% glutamine, penicillin, and streptomycin. The purity of the sorted population was 95–99%. Murine L cells transfected with human B7.1/CD80 (Dap/B7), HLA-DRB1*0101 (5-3.1), and 5-3.1 cotransfected with B7.1/CD80 (Dap/B7), HLA-DRB1*0101 (5-3.1), and 5-3.1 cotransfected with B7.1/CD80 (5-3.1/B7) were previously described (31, 32). The following Abs were used: mouse anti–NF-ATc1, goat anti-PIPKαα (N-20), goat anti-PIPKαα (C17), mouse anti-α-tubulin, rabbit anti-phosPHOLC1y (Tyr785), rabbit anti-PLCγ1, and mouse anti-hemagglutinin (HA; Santa Cruz Biotechnology); and mouse anti-CD28.2, goat anti-mouse (GAM [Amersham]), and mouse anti-CD3 (UCHT1) (BD Pharmingen). Staphylococcal enterotoxin A (SEA), neuromycin, PI4, SP2, and PI4P were purchased from Sigma-Aldrich (Milan, Italy); staphylococcal enterotoxin E (SEE) and staphylococcal enterotoxin B (SEB) were from Toxin Technology (Sarasota, FL). The PI3K inhibitor AS-605240 was purchased from Cayman Chemical.

Plasmids and cell transfection
HA-tagged kinase-dead mutant PIPKαα (D310K) mutated in the highly conserved DAKRP sequence within the kinase core domain (25) was generated by PCR from the cDNA of human primary CD4+ T cells. Briefly, wild-type PIPKαα was amplified using oligonucleotides containing N-terminal Nhel and C-terminal NotI restriction sites for cloning in pcDNA-HA expression vector. D310K substitution was introduced into wild-type PIPKαα by two-step PCR mutagenesis with the following oligonucleotides: 5′-AGCTTCAAGATATGGAATAGCCTTGG-3′ and 5′-CAAGGGCTATCCATATTATGGAAGCT-3′. The PCR products were then coamplified with PIPKαα coding sequence 5′ and 3′ primers with the following Nhel and NotI restriction sites (TGATGACCCGTCGCCCCTCCTCCCGG-3′ and 5′-TGGGCGCGCATGGTGAACTGACTC-3′). The entire sequence of HA-PIPKαα (D310K) construct was verified by DNA sequencing.

Primary CD4+ T cells, resuspended in 100 µl Nucleofector solution (Amaxa Biosystems), were electroporated with the indicated expression vector using the V-024 program of the Nucleofector.

RNA interference
Primary CD4+ T cells were transfected with 5 µg pooled selection-designed siRNAs (UCAGUGAGGGCGCGCUA; UCUAGCGGUGUUGAGAGUUA; Dharmacron, Lafayette, CO) for PIPKαα or with 5 µg scrambled control siRNAs, using Amaxa Nucleofector kit. Cells were then incubated in complete medium for 72 h before harvesting, and the level of PIPKαα silencing was analyzed by Western blotting.

Cell stimulation and immunoblotting
Primary CD4+ T cells were stimulated as indicated at 37°C. At the end of incubation, cells were harvested and lysed for 30 min on ice in 1% Nonidet P-40 lysis buffer in the presence of inhibitors of proteases and phosphatases. Extracts were precleared for 1 h with protein G-Sepharose and then immunoprecipitated for 2 h with anti-CD28 Abs and activated PIPKαα, followed by anti-PI4P, as previously described (33). Briefly, PI4P was resuspended in assay buffer (30 mM HEPES [pH 7.4], 100 mM KCl, 1 mM EGTA, 2 mM MgCl2, 0.05% Nonidet P-40) and sonicated. Beads containing PIPKαα were incubated with 50 µl assay buffer containing PI4P, MgATP (50 µM), and ATP γ -ATP for 15 min at 30°C. The organic phase, containing PIP2, was separated by thin-layer chromatography on Silica gel 20 × 10 mm plates (Merck, Darmstadt, Germany). The radioactive lipids were visualized by autoradiography. The identity of PIP2 and PI4P was confirmed by comparison with standard phospholipids revealed by iodine vapor. The spot corresponding to PIP2 was quantified by densitometric analysis using the ImageJ program (National Institutes of Health).

To measure the amount of PIP2 in primary CD4+ T cells stimulated by anti-CD3 or anti-CD28, a nonradioactive competitive ELISA kit was used (Echelon Biosciences). Briefly, CD4+ T cells were stimulated for 5 min with anti-CD28 (5 µg/ml) or anti-CD3 (5 µg/ml) Abs cross-linked by GAM (20 µg/ml); PIP2 lipids were extracted by chloroform/methanol/HCl and measured, as indicated (35).

Confocal microscopy
A total of 15 × 10^5 Dap/B7, or 5-3.1, or 5-3.1/B7 cells preincubated with a mixture of superantigen (SEE, SEA, and SEB, 1 µg/ml each) was adhered on cover glasses (12 mm) overnight at 37°C. Primary CD4+ T cells (5 × 10^5), transfected for 24 h with NF-ATc (1 µg) or NF-ATc together with 1 µg kinase-dead HA-PIPKαα (D310K), were then seeded on cover glasses for 3 h, whereas T cells transfected for 24 h with GFP-PIPKαα were seeded on cover glasses for 15 min at 37°C, fixed by 2% paraformaldehyde, and permeabilized by 0.1% saponin in PBS containing 1% BSA. For the experiments with anti-CD28 or anti-CD3 Abs, primary CD4+ T cells were cotransfected at 1:3 ratios with sulfated latex microspheres (Molecular Probes, Invitrogen) coated with anti-CD28 or anti-CD3 plus anti-CD28 Abs before seeding on cover glasses. Cells were then fixed by 2% paraformaldehyde and permeabilized by 0.1% saponin in PBS containing 1% BSA. NF-ATc was stained using anti–NF-ATc1 Abs, followed by Alexa Fluor 488–conjugated GAM serum. Nucleus was stained by 0.1 mg/ml propidium iodide. Confocal observations were performed using a Leica DMIRE apparatus (Leica Microsystems, Heidelberg, Germany) equipped with an argon-krypton laser, double-dichroic splitters (488/568 nm). Image acquisition and processing were conducted by using the Leica confocal software (Leica LCS). Images were analyzed with the Adobe Photoshop 7.0 program. The relative recruitment index (rRI) was calculated, as previously described, by the following formula: rRI = (mean fluorescence intensity [MFI] at synapse − background)/MFI at all the cell membrane restriction sites (not in contact with synapse). At least 15 cells or conjugates were examined quantitatively for each experimental significance. Statistical significance was calculated using Student t-test. Signals from different fluorescent probes were taken in parallel. Several cells were analyzed for each labeling condition, and representative results are presented.

Measurement of intracellular calcium concentration
Primary CD4+ T cells (1.5 × 10^5/ml) were loaded with 20 µM fluo-3-acetoxyethyl ester (Sigma-Aldrich) for 30 min at 37°C in 300 µl RPMI (1640) loaded cells were then washed and actin was expressed. CD3 plus anti-CD28 Abs cross-linked with GAM at 37°C and immediately analyzed by a cytofluorimeter (FACScalibur; BD Biosciences). Changes in cell fluorescence were monitored every 24 s for 10 min by measuring fluorescence emission at 530 nm. The concentration of intracellular calcium [Ca2+] was calculated according to (36).

Real-time PCR
Total RNA was extracted using RNeasy MicroKit (Qiagen) from 5 × 10^5 CD4+ T cells and was reverse transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase (Invitrogen). TaqMan Universal PCR Master Mix, IL-8, IL-2, and GAPDH primer/probe sets were purchased from Applied Biosystems. The relative quantification was performed using the comparative cycle threshold method.
Statistical analysis

Statistical analyses were performed with Microsoft Excel software using the Student t test. Differences were assumed significant when \( p < 0.05 \).

Results

CD28-mediated activation of PI3K regulates PIP2 turnover in primary CD4\(^+\) T cells

One well-established mediator of several CD28 functions is the PI3K, which is activated following the direct recruitment of the p85 adaptor subunit to the highly conserved YMMM motif in CD28 cytoplasmic tail (4, 37, 38). PI3K acts upon membrane phosphoinositides, and PI3P2 is a crucial substrate in regulating the signaling pathway downstream to PI3K (6). PI3K turnover may be an essential limiting factor ensuring the activation of PI3K following CD28 engagement; we therefore measured PIP2 levels in primary CD4\(^+\) T lymphocytes stimulated with anti-CD28 or anti-CD3 Abs. We found that CD28 triggering alone induced a strong reduction of PIP2 levels, almost comparable to the rate of PIP2 consumption induced by TCR stimulation (Fig. 1A). Because PIP2 turnover requires new synthesis to provide sufficient substrate in response to cellular receptors (30) and CD28-mediated PI3K activation utilizes PIP2 (39), we quantified PIP2 by inhibiting PI3K activity. CD28-induced variation of PIP2 was measured in the presence of the PI3K inhibitor AS-605240. As positive control, we also used neomycin, an aminoglycoside that strongly binds PIP2 and makes it unavailable to several enzymes (40). The inhibition of PI3K activity strongly increased PIP2 over the basal amount in CD28-stimulated cells at a level comparable to those induced by neomycin treatment (Fig. 1B).

These results demonstrate that CD28 regulates the rate of PIP2 synthesis in primary CD4\(^+\) T cells. **

CD28 recruits and activates PIP5K\(α\)

The local synthesis of PIP2 is mainly regulated by PIP5Ks (30). In this study, we tested the possibility that CD28 might be coupled to the activation of PIP5K\(α\). We first performed a time course analysis of PIP5K\(α\) kinase activity in response to CD28 stimulation of CD4\(^+\) T lymphocytes. CD28 stimulation with agonistic Abs strongly upregulated PIP5K\(α\) activity, which increased after 5 min of stimulation and started to decrease after 10–20 min (Supplemental Fig. 1). We next compared PIP5K\(α\) activity in response to CD28 and/or CD3 stimulation. Endogenous PIP5K\(α\) was immunoprecipitated from primary CD4\(^+\) T cells either unstimulated or stimulated for 5 min with anti-CD28, or anti-CD3, or anti-CD3 plus CD28 Abs, and its kinase activity was evaluated (Fig. 2A). CD28 engagement by its natural ligand B7.1/CD80 was also investigated by using adherent Dap/B7 cells. We have previously demonstrated that this system perfectly mimics the physiological CD28/CD80 encounter (33, 41–43). Primary T cells were stimulated for 5 min with adherent Dap/B7 cells. At the end of stimulation, T cells were carefully harvested to avoid detachment of adherent cells. The FAC5 analysis of CD3 and CD28 expressions revealed that the recovered T cell population was 95% pure. CD28 stimulation by both B7.1/CD80 and an agonistic anti-CD28 Ab strongly increased PIP5K\(α\) kinase activity (lanes 3 and 4, versus lane 2) at a level comparable to that induced by anti-CD3 stimulation alone (lane 4 versus lane 5). No cooperative increase of PIP5K\(α\) activity was observed when CD3 and CD28 were coengaged (lane 6 versus lanes 5 and 3). Fig. 2B shows the densitometric analysis of kinase activity expressed as fold induction normalized to PIP5K\(α\) levels.

We then explored the mechanisms responsible for CD28-induced PIP5K\(α\) activation by addressing whether CD28 stimulation recruits PIP5K\(α\) to the plasma membrane. Because the anti-PIP5K\(α\) Abs exhibited low binding affinity, we performed these analyses by transfecting CD4\(^+\) T cells with GFP-PIP5K\(α\). T cells were stimulated for 15 min with B7.1/CD80-positive (Dap/B7) or B7.1/CD80-negative (5-3.1) cells. Confocal microscopy analyses revealed that CD28/CD80 interaction by itself efficiently induced the recruitment of PIP5K\(α\) to the plasma membrane in the T:APC contact zone (Fig. 2C, 2D). Thus, CD28 stimulation recruits and activates PIP5K\(α\).

**PI5K\(α\) silencing impairs CD28-mediated IL-8 and IL-2 transcriptional activation**

We next investigated the contribution of PIP5K\(α\) to either CD28 unique signals or CD28/TCR costimulatory signals. In particular, we looked at IL-8 gene transcription, which we have previously demonstrated to be a specific target of CD28, but not TCR (33), and at IL-2 gene. PIP5K\(α\) silencing significantly impaired (50%) IL-8 gene expression induced by CD28 individual ligation (Fig. 3A). The analysis of siRNA efficiency, evaluated by Western blotting, evidenced that >80% of endogenous PIP5K\(α\) was knocked down (Fig. 3B). Consistent with the key role of CD28 signals in inducing IL-2 gene expression (17, 44, 45), an efficient IL-2 transcription was achieved following TCR and CD28 coengagement (Fig. 3C). IL-2 gene expression was also greatly downregulated in silenced cells, demonstrating a critical role of PIP5K\(α\) in IL-2 transcriptional signals.

PI5K\(α\) silencing impairs CD28-mediated costimulatory signals necessary for sustaining Ca\(^{2+}\) release

To assess whether PIP5K\(α\) could play a role in CD28-dependent sustained Ca\(^{2+}\) wave, primary CD4\(^+\) T cells were transfected with
PIP5Kα siRNA, and Ca²⁺ influx was measured following anti-CD3 plus anti-CD28 stimulation. According to the crucial role of CD28 in amplifying TCR signals necessary for sustained Ca²⁺ influx (46), CD28 coengagement induced a stronger and prolonged Ca²⁺ elevation compared with CD3 individual ligation (Fig. 4A). PIP5Kα silencing strongly impaired the increment of Ca²⁺ levels mediated by CD28 costimulation. Moreover, the inhibition of Ca²⁺ influx was not due to the impairment of TCR/CD28-mediated activation of PLCγ₁, as demonstrated by the comparable levels of PLCγ₁ Tyr783 phosphorylation observed in TCR/CD28-stimulated cells transfected with control (Scr) or PIP5Kα siRNA (Fig. 4C, lane 4 versus lane 2).

These results indicate that CD28-mediated PIP5Kα activation regulates Ca²⁺ signaling pathway in primary T cells.

**FIGURE 3.** PIP5Kα is required for CD28-mediated transcription of both IL-8 and IL-2 genes. (A) Primary CD4⁺ T cells were transfected with PIP5Kα siRNA or scrambled control siRNA (scr siRNA) for 72 h and then stimulated for 6 h with anti-CD28 Abs. IL-8 mRNA levels were measured and expressed as arbitrary units (AU). Bars show the mean ± SD of three independent experiments. *p < 0.01, **p < 0.05 calculated by Student t test, compared with unstimulated or CD28-stimulated cells transfected with scr siRNA, respectively. (B) The efficacy of PIP5Kα silencing of one representative experiment is shown. F.I. = fold inductions. (C) Real-time PCR was used to measure IL-2 mRNA levels in CD4⁺ T cells transfected as in (A) and stimulated for 6 h with cross-linked anti-CD3 or anti-CD3 plus anti-CD28 Abs. Data are expressed as arbitrary units (AU). Bars show the mean ± SD of three independent experiments. *p < 0.01 calculated by Student t test, compared with anti-CD3 plus anti-CD28–stimulated cells transfected with scr siRNA.
The kinase activity of PIP5Kα regulates NF-AT nuclear translocation and IL-2 gene expression in CD28-costimulated T cells

The selective synthesis of PIP2 by PIP5K depends on a kinase core domain with conserved catalytic residues (25, 47). To verify a possible involvement of the kinase activity of PIP5Kα in TCR/CD28-dependent Ca2+ signaling cascade, we generated a kinase-dead mutant of PIP5Kα by substituting Asp310 with Lys (D310K). This residue is located in DAKRP sequence within the kinase core domain that is highly conserved in all PIP5K isoforms (25), and its mutation leads to a lipid-kinase defective mutant with dominant-negative functions in several cell types (48–50). Primary CD4+ T cells were transfected with NF-ATc expression construct alone or together with kinase-dead PIP5Kα (D310K) vector, and NF-ATc subcellular distribution was analyzed following stimulation with sulfate latex microspheres coated with optimal concentration of anti-CD28 or anti-CD3 plus anti-CD28 Abs (Fig. 5A). Approximately 70% of T cells showed a significant nuclear translocation of NF-ATc, when TCR and CD28 were coengaged (Fig. 5B). In contrast, >70% of T cells expressing kinase-dead PIP5Kα (D310K) mutant upon stimulation showed a cytoplasmic NF-ATc localization. No nuclear translocation of NF-AT was observed when T cells were stimulated with microspheres coated with isotype-matched IgG or anti-CD28 Abs. Similar results were obtained by stimulating primary T cells with adherent 5-3.1/B7, or 5-3.1/B7 cells pulsed with a mixture of superantigens SEE, SEA, and SEB (Supplemental Fig. 2). Consistent with these results, the overexpression of kinase-dead PIP5Kα (D310K) mutant strongly inhibited IL-2 transcription induced by TCR plus CD28 stimuli (Fig. 5D).

All together these evidences indicate that PIP5Kα activity is required to ensure optimal TCR- and CD28-induced IL-2 gene expression.

Discussion

PIP2 represent <1% of plasma membrane phospholipids; a balanced PIP2 consumption and replenishment have been described in response to receptor stimulation (30). The activity and localization of PIP5Ks are essential for the production of PIP2, which plays a critical role in the regulation of both cytoskeleton dynamics and second messenger generation. Primary T cells express all three PIP5K isoforms (51), each presenting a specific subcellular localization that provides both temporally and spatially regulated distinct pool of PIP2. For instance, human PIP5Kα (the ortholog of murine PIP5Kβ) localizes primarily to the plasma membrane, and, at membrane ruffles, PIP5Kβ has been observed in nuclear and perinuclear vesicles (52) and PIP5Kγ was reported to localize to intracellular membrane compartment (49) as well as to focal adhesion plaques (53). Furthermore, data obtained by overexpressing PIP5Kα, β, and γ isoforms in transgenic mice stimulated by Ag evidenced the enrichment of different PIP5K isoforms at the IS (51), which was also described as the site of most intense PIP2 turnover (22). To our knowledge, the coupling of CD28 to PIP5Ks and PIP2 metabolism remains unknown. In this study, we show that CD28 represents a key node in the activation of PIP5Kα and in the regulation of PIP2 turnover.

PIP2 is the common source for two major distinct signaling cascades involving PI3K and PLCγ1, which often colocalize in the same signaling complexes competing for the common pool of substrate. Consequently, PIP2 levels decrease following receptor activation, and the replenishment of a PIP2 pool may be an essential regulatory step to ensure the activation of both PI3K and PLCγ1. PI3K contributes to the local consumption of PIP2 by converting it to PI(3,4,5)P3 (54). PI3P lipids bind pleckstrin homology domains of several molecules involved in T cell activation. Although TCR stimulation has been shown to induce PI3K activation (55), CD28 is known to give a major contribution in activating PI3K pathway (56); indeed, CD28 recruits and activates class I PI3K (39) in a TCR-independent manner (56). Our results evidence that, by recruiting and activating PIP5Kα (Fig. 2), CD28 may contribute to increase the synthesis and turnover of PIP2.
FIGURE 5. Overexpression of kinase-dead PIP5Kα (D310K) mutant impairs NF-AT nuclear translocation and IL-2 gene expression in TCR/CD28-stimulated T cells. (A) Primary CD4+ T cells were transfected for 24 h with NF-ATc construct (1 μg) together with control empty vector (Vec) or kinase-dead HA-PIP5Kα (D310K) construct (1 μg) and then stimulated at 1:3 ratio for 3 h with sulfate latex microspheres coated with isotype-matched mouse IgG, or mouse anti-CD28 (5 μg/ml) or mouse anti-CD3 plus anti-CD28 (5 μg/ml each) Abs. After fixing, cells were stained with propidium iodide (PI) to visualize nucleus (red), and NF-ATc intracellular localization (green) was evaluated by confocal microscopy after staining with anti-NF-ATc1, followed by Alexa Fluor 488–conjugated anti-mouse Ab. In this way, the beads coated with the Abs were also stained. Each image is representative of 15 heterocaryons analyzed for each coverslip. Scale bar, 10 μm. (B) The percentage of cells with NF-AT nuclear translocation was calculated and represents the mean ± SD of three independent experiments. **p < 0.05 calculated by Student t test compared with anti-CD3– plus anti-CD28–stimulated cells transfected with empty vector. (C) The expression of HA-PIP5Kα (D310K) was analyzed by anti-HA Western blotting. All data are representative of three independent experiments. (D) Real-time PCR was used to measure IL-2 mRNA levels in CD4+ T cells transfected for 24 h with control empty vector (Vec) or kinase-dead HA-PIP5Kα (D310K) construct (2 μg) and stimulated for 6 h with cross-linked anti-CD3 plus anti-CD28 Abs. Data are expressed as fold inductions over the basal level of unstimulated cells. Bars show the mean ± SD of three independent experiments. **p < 0.05 calculated by Student t test, compared with anti-CD3– plus anti-CD28–stimulated cells transfected with empty vector.

The activation of the NF-κB pathway and NF-κB-regulated genes is a unique feature of CD28. Stimulation of T cells with either professional APCs or anti-TCR plus anti-CD28 Abs efficiently activates NF-κB, whereas TCR alone resulted in being unable to do that (15, 57). The transcription factors of Rel/NF-κB family are critical regulators of the immune system by controlling both innate and adaptive immune responses and the expression of >150 inflammatory cytokines and chemokines (58). We have previously demonstrated that, in effector/memory CD4+ T cells, CD28 engagement by agonistic Abs or B7.1/CD80 expressed on the surface of APCs in the absence of TCR leads to the activation of an alternative NF-κB2–like cascade and to the nuclear translocation of p52/RelA dimers, which in turn selectively regulate the expression of proinflammatory cytokines/chemokines, such as IL-8 (33, 41, 42). We further extend these previous data by demonstrating that CD28-activated PIP5Kα is essential for NF-κB–dependent IL-8 expression (Fig. 3A).

CD28 also amplifies and sustains TCR-dependent PLCγ1-mediated hydrolysis of PIP2 (59). This event leads to the generation of IP3 as messenger that mediates the release of Ca2+ from the intracellular stores. This initial wave is sustained by CD28 (46) and is necessary for Ca2+ influx through the gating of membrane CRAC channels, which represent the major Ca2+ influx pathway in T cells (60, 61). Recently, in both humans and mice, PIP5Kα isoform has been shown to recruit the plasma membrane in response to several receptors, where it provides the substrate PIP2 for PLCγ1, thus inducing IP3 formation and Ca2+ mobilization (27–29). Indeed, in silenced cells (Fig. 4) as well as in cells overexpressing a kinase-dead mutant of PIP5Kα (Fig. 5), we observed a strong inhibition of CD28-costimulated Ca2+ influx (Fig. 4A), NF-AT nuclear translocation (Fig. 5A, 5B), and IL-2 gene transcription (Figs. 3C, 5D).

The decreased response is not associated with the impairment of TCR/CD28-induced tyrosine phosphorylation of PLCγ1 (Fig. 4C), indicating that the observed effects depend on a defective PIP2 refilling.

Recent data by Sun et al. (51) also evidenced that PIP2 synthesis occurs at the T:APC interface. Spatiotemporal analysis of both PIP2 distribution and turnover evidenced that PIP2 concentrates at the IS very early during Ag recognition, where it is rapidly hydrolyzed by PLCγ1 or phosphorylated by PI3K (22). Our data extend these previous reports demonstrating an important contribution of CD28 in regulating PIP5Kα activation and PIP2 turnover (Figs. 1, 2) in human T lymphocytes. Interestingly, a significant pool of PIP2 associates with membrane rafts (62, 63), which serve as membrane platforms for efficient TCR signaling (64). Thus, through the activation of PIP5Kα, CD28 may contribute to the enrichment of PIP2 in rafts, resulting in the augmentation of PIP2-dependent production of second messengers (65, 66). Alternatively, because both PIP2 and PIP5Kα (23) as well as CD28 have been involved in actin polymerization (67), CD28-mediated recruitment and activation of PIP5Kα may promote the cytoskeleton reorganization events necessary for the redistribution of key signaling molecules at the membrane. Interestingly, our preliminary data suggest that the C-terminal proline-rich motif of CD28, which is critical for the recruitment of the actin-binding protein filamin A (65), the accumulation of lipid rafts (68), and NF-κB activation (42), is also implicated in PIP5Kα recruitment (Supplemental Fig. 3).

By ensuring PIP2 availability essential for the activation of PI3K/Akt, sustained Ca2+ influx, NF-AT nuclear translocation, and the expression of pivotal genes, we identified PIP5Kα as a critical regulator of multiple CD28-mediated signaling functions in T lymphocytes. Our results also highlight a previously unknown role of CD28 in refilling the PIP2 pool necessary to ensure optimal T cell activation. The characterization of the molecular mechanisms and
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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1.

Time course analysis of PIP5Kα kinase activity in CD28-stimulated CD4⁺ T cells. (A) CD4⁺ T cells were unstimulated (-) or stimulated for the indicated times with saturating concentration of anti-CD28 (5 μg/ml) crosslinked with GAM (20 μg/ml). PIP5K kinase assays were performed on anti-PIP5Kα immunoprecipitations (IP) and the reaction products were subjected to TLC followed by autoradiography (upper panel). An equal amount of cell lysate was analyzed for PIP5Kα content by Western blotting (lower panel). (B) Fold inductions were quantified by densitometric analysis and normalized to PIP5Kα levels. The results are representative of three independent experiments.
Supplemental Figure 2.
Overexpression of kinase dead PIP5Kα (D310K) mutant impairs TCR and CD28-mediated NF-AT nuclear translocation in CD4+ T cells. (A) Primary CD4+ T cells were transfected for 24 h with NF-ATc construct (1 μg) together with control empty vector (Vec) or kinase dead HA-PIP5Kα (D310K) construct (1 μg) and then stimulated for 3 h with adherent 5-3.1/B7, or 5-3.1/B7 cells pulsed with a cocktail of superantigens (SAGs) SEE, SEA and SEB (1μg/ml each). After fixing cells were stained with propidium iodide (PI) to visualize nucleus (red) and NF-ATc intracellular localization (green) was evaluated by confocal microscopy after staining with anti-NF-ATc1 followed by AlexaFluor488 conjugated anti-mouse Ab. Each image is representative of 15 heterocaryons analyzed for each coverslip. Scale bar: 10 μm. (B) The % of cells with NF-AT nuclear translocation was calculated and represents the mean ± SD of three independent experiments. Asterisks (*) and (**) indicate P <0.01 and P <0.05 calculated by Student’s t test compared with cells transfected with empty vector and stimulated with unpulsed 5-3.1/B7 or SAGs-pulsed 5-3.1/B7 cells, respectively.
Supplemental Figure 3.
The C-terminal proline-rich motif of CD28 is involved in PIP5Kα recruitment. (A) CD28-negative Jurkat T cells (IC7C17) stably transfected with human wild-type CD28 (CD28 WT), or with CD28 mutants in the Y<sup>191</sup>MNM motif (CD28<sup>Y<sup>191</sup>F</sup>) or in the C-terminal proline-rich Y<sup>208</sup>QP<sup>208</sup>YAPP motif (CD28-3A), were transfected for 24 h with GFP-PIP5Kα construct and then stimulated with adherent Dap/B7. After fixing by 2% paraformaldehyde, cells were analysed by confocal microscopy. Scale bar: 10 μm. (B) The relative recruitment index (RRI) was calculated as described in Materials and Methods and represents the mean ± SD of 15 conjugates analyzed in each group. Asterisk (*) indicate P < 0.01 calculated by Student’s t test compared with CD28 WT cells transfected with GFP-PIP5Kα and left unstimulated.