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

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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 activation is required for both CD28 unique signals regulating IL-8 gene expression as well as for CD28/TCR-induced Ca^{2+} mobilization, NF-AT nuclear translocation, and IL-2 gene transcription. Our findings elucidate a novel mechanism that involves PIP5Kα as a key modulator of CD28 costimulatory signals. ***The Journal of Immunology*****, 2013, 190: 5279–5286.**

CD28 costimulatory receptor is a crucial determinant of the outcome of T lymphocyte activation. The engagement of CD28 by its natural ligands, 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 YMNM 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) lipids (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 PIP2 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 PI3K (22). The main biosynthetic pathway of PIP2 involves phosphorylation of phosphatidylinositol 4-monophosphate (PI4P) at the D5 position of the inositol ring by phosphatidylinositol 4-phosphate 5-kinases (PIP5K) (23). Three PIP5K isoforms (α, β, and γ) have been identified (24–26). Although the visualization of endogenous PIP5K isoforms has been often difficult, several data obtained in different cell systems evidenced differential subcellular localizations of each isoform.

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Abbreviations used in this article: $[Ca^{2+}]_i$, intracellular calcium; GAM, goat anti-mouse; HA, hemagglutinin; IP3, inositol trisphosphate; IS, immunological synapse; PDK1, phosphoinositide-dependent kinase 1; PI4P, 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 interference RNA.

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PIP5Kα, 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 PIP5Kα in human primary $CD4^+$ T lymphocytes. This event leads to the neosynthesis of PIP2 that is consumed by CD28-activated PI3K. By either small interference RNA (siRNA)–driven cell silencing or overexpressing a kinase-dead mutant, we evidenced that PIP5Kα activation is required for both CD28 autonomous signals regulating IL-8 gene expression as well as for CD28/TCR-induced Ca^{2+} mobilization, NF-AT nuclear translocation, and IL-2 gene transcription. Our findings identify PIP5Kα 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, Milano, Italy) and cultured in RPMI 1640 supplemented with 5% human serum (Euroclone, UK), L-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 (5-3.1/B7) were previously described (31, 32). The following Abs were used: mouse anti–NF-ATc1, goat anti-PIP5Kα (N-20), goat anti-PIP5Kα (C17), mouse anti-α tubulin, rabbit anti-phosphoPLCγ1 (Tyr^{783}), 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), neomycin, PI4,5P2, 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 PIP5Kα (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 PIP5Kα was amplified using oligonucleotides containing N-terminal NheI and C-terminal NotI restriction sites for cloning in pcDNA-HA expression vector. D310K substitution was introduced into wild-type PIP5Kα by two-step PCR mutagenesis with the following oligonucleotides: 5′-AGCTTCAAGATAATGAAGTATAGCCTCTTG-3′ and 5′-CAAGAGGCTATACTTCATTATCTTGAAGCT-3′. The PCR products were then coamplified with PIP5Kα coding sequence 5′ and 3′ primers with the following NheI and NotI restriction sites: 5′-TTGCTAGCGCGTCGGCCTCCTCCGGG-3′ and 5′-TTGCGGCCGCATGGGTGAACTCTGACTC-3′. The entire sequence of HA-PIP5Kα (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 (UCAGUGAGGGCUCGCCUA; UCUACGUGGUUGAGAGUAU; Dharmacon, Lafayette, CO) for PIP5Kα 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 PIP5Kα 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-CD28.2 preadsorbed on protein G–Sepharose beads (Amersham). Nuclear extracts were prepared, as previously described (33). Proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with the indicated primary Abs, extensively washed, and, after incubation with HRP-labeled goat anti-rabbit or HRP-labeled GAM, developed with the ECL's detection system (Amersham).

PIP5K kinase assay and measurement of PIP2 levels

Primary $CD4^+$ T cells (20×10^6) were stimulated for 5 min with adherent Dap/B7 cells, or anti-CD28 (5 μg/ml), or anti-CD3 (5 μg/ml) Abs cross-linked with 20 μg/ml GAM. 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 PIP5Kα was immunoprecipitated and incubated with PI4P, as previously described (34). Briefly, PI4P was resuspended in assay buffer (30 mM HEPES [pH 7.4], 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 0.05% Nonidet P-40) and sonicated. Beads containing PIP5Kα were incubated with 50 μl assay buffer containing PI4P, MgATP (50 μM), and $^{32}\gamma$-ATP for 15 min at 30°C. The organic phase, containing PIP2, was separated by thin-layer chromatography on Silica gel 20×10 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 15×10^3 Dap/B7, or 5-3.1, or 5-3.1/B7 cells preincubated with a mixture of superantigens (SEE, SEA, and SEB, 1 μg/ml each) was adhered on cover glasses (12 mm) overnight at 37°C. Primary $CD4^+$ T cells (150×10^3), transfected for 24 h with NF-ATc (1 μg) or NF-ATc together with 1 μg kinase-dead HA-PIP5Kα (D310K), were then seeded on cover glasses for 3 h, whereas T cells transfected for 24 h with GFP-PIP5Kα 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 conjugated at 1:3 ratio with sulfate 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 not in contact with APC − background). At least 15 cells or conjugates were examined quantitatively for each experiment. Statistical significance was calculated using a 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 (1.5×10^6/ml) were loaded with 20 μM fluo-3-acetoxymethyl ester (Sigma-Aldrich) for 30 min at 37°C in 300 μl RPMI 1640. Loaded cells were then washed and activated with anti-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 $[Ca^{2+}]_i$ 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 YMNM motif in CD28 cytoplasmic tail (4, 37, 38). PI3K acts upon membrane phosphoinositides, and PIP2 is a crucial substrate in regulating the signaling pathway downstream to PI3K (6). PIP2 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.

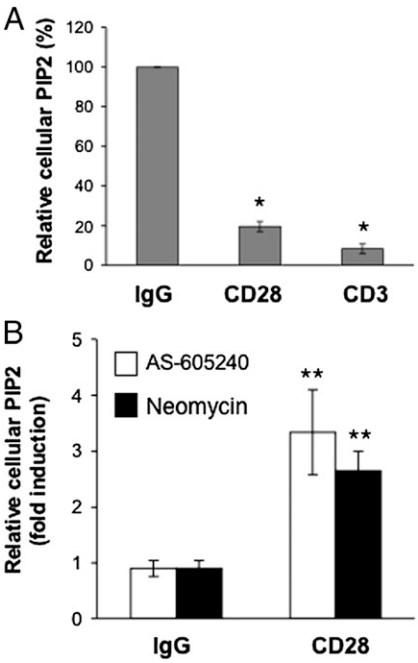

FIGURE 1. CD28 stimulation regulates PIP2 turnover in primary CD4$^+$ T cells. (**A**) Biochemical quantification of PIP2 levels extracted by 5×10^6 CD4$^+$ T cells stimulated for 5 min with control isotype-matched mAb (IgG), or anti-CD28 (5 μg/ml), or anti-CD3 (5 μg/ml) Abs. PIP2 value of control mAb-stimulated cells was assumed as 100%. Data express the mean ± SD of three independent experiments. *$p < 0.01$ calculated by Student *t* test compared with controls. (**B**) CD4$^+$ T cells were stimulated with control isotype-matched mAb (IgG) or anti-CD28 Abs in the presence of AS-605240 (10 μM) or neomycin (5 mM). PIP2 value of unstimulated cells was assumed as 1, and fold inductions over the basal level were calculated. Data express the mean ± SD of three independent experiments. **$p < 0.05$ calculated by Student *t* test, compared with unstimulated cells.

CD28 recruits and activates PIP5Kα

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 PIP5Kα. We first performed a time course analysis of PIP5Kα kinase activity in response to CD28 stimulation of CD4$^+$ T lymphocytes. CD28 stimulation with agonistic Abs strongly upregulated PIP5Kα activity, which increased after 5 min of stimulation and started to decrease after 10–20 min (Supplemental Fig. 1). We next compared PIP5Kα activity in response to CD28 and/or CD3 stimulation. Endogenous PIP5Kα 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/B7 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 FACS 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 PIP5Kα 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 PIP5Kα 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 PIP5Kα levels.

We then explored the mechanisms responsible for CD28-induced PIP5Kα activation by addressing whether CD28 stimulation recruits PIP5Kα to the plasma membrane. Because the anti-PIP5Kα Abs exhibited low binding affinity, we performed these analyses by transfecting CD4$^+$ T cells with GFP-PIP5Kα. 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 PIP5Kα to the plasma membrane in the T:APC contact zone (Fig. 2C, 2D). Thus, CD28 stimulation recruits and activates PIP5Kα.

PIP5Kα silencing impairs CD28-mediated IL-8 and IL-2 transcriptional activation

We next investigated the contribution of PIP5Kα 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. PIP5Kα 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 PIP5Kα 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 PIP5Kα in IL-2 transcriptional signals.

PIP5Kα silencing impairs CD28-mediated costimulatory signals necessary for sustaining Ca^{2+} release

To assess whether PIP5Kα could play a role in CD28-dependent sustained Ca^{2+} wave, primary CD4$^+$ T cells were transfected with

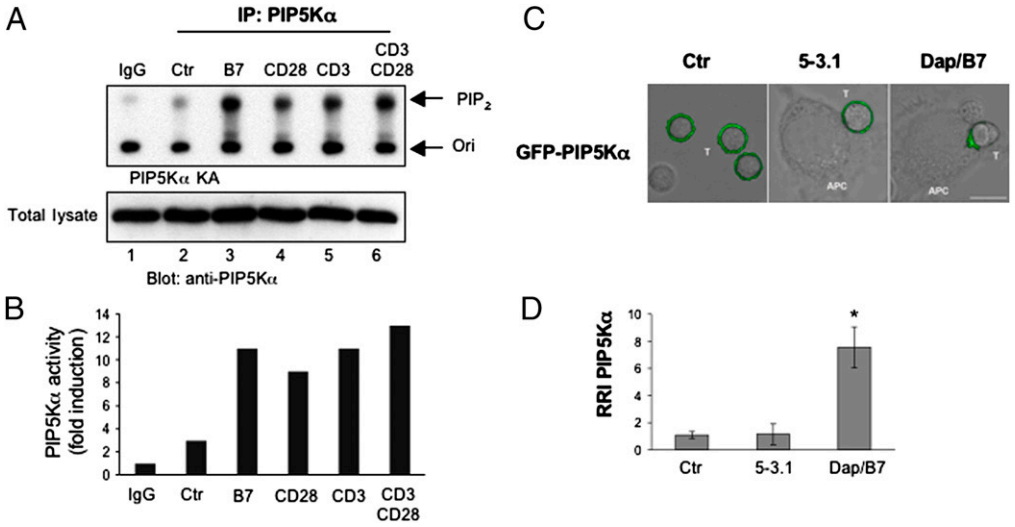

FIGURE 2. CD28 stimulation induces the membrane recruitment and activation of PIP5Kα in primary CD4$^+$ T cells. (**A**) CD4$^+$ T cells were left unstimulated (ctr) or stimulated for 5 min with adherent Dap/B7 cells (B7), or saturating concentration of anti-CD28 (5 μg/ml), or anti-CD3 (5 μg/ml), or anti-CD3 plus anti-CD28 Abs cross-linked with GAM (20 μg/ml). PIP5K kinase assays were performed on anti-PIP5Kα or isotype-matched control mAb (IgG) immunoprecipitations (IPs), and the reaction products were subjected to thin-layer chromatography, 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. (**C**) Primary CD4$^+$ T cells were transfected with GFP-PIP5Kα construct (2 μg) for 24 h and then stimulated for 15 min with adherent murine L cells (5-3.1) or murine L cells transfected with human B7.1/CD80 (Dap/B7). After fixing, cells were analyzed by confocal microscopy. The scale bar represents 10 μm. (**D**) 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. $*p < 0$ 0.01 calculated by Student *t* test compared with controls. More than 70% of T:Dap/B7 conjugates showed PIP5Kα RRI > 5. The results are representative of three independent experiments.

PIP5Kα siRNA, and Ca^{2+} influx was measured following anti-CD3 plus anti-CD28 stimulation. According to the crucial role of CD28 in amplifying TCR signals necessary for sustained Ca^{2+} influx (46), CD28 coengagement induced a stronger and prolonged Ca^{2+} elevation compared with CD3 individual ligation (Fig. 4A). PIP5Kα silencing strongly impaired the increment of Ca^{2+} levels mediated by CD28 costimulation. Moreover, the inhibition of Ca^{2+} influx was not due to the impairment of TCR/CD28-mediated activation of PLCγ1, as demonstrated by the comparable levels of PLCγ1 Tyr^{783} 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 Ca^{2+} signaling pathway in primary T cells.

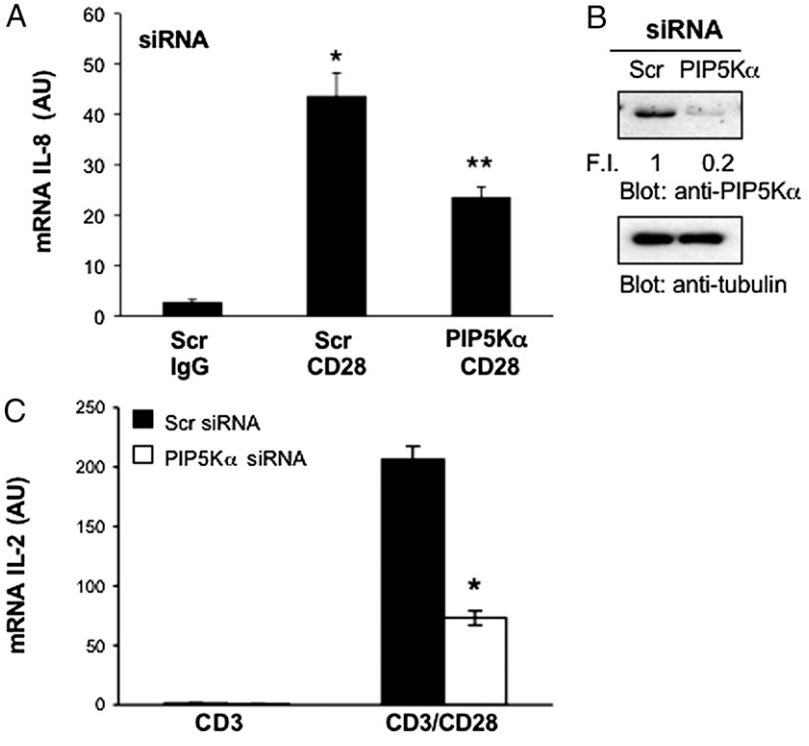

FIGURE 3. PIP5Kα is required for CD28-mediated transcription of both IL-8 and Il-2 genes. (**A**) Primary CD4$^+$ T cells were transfected with PIP5Kα siRNA or scrambled control siRNA (scr siRNA) for 72 h and then stimulated for 6 h with anti-CD28 Abs. IL-8 mRNA levels were measured and expressed as 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.

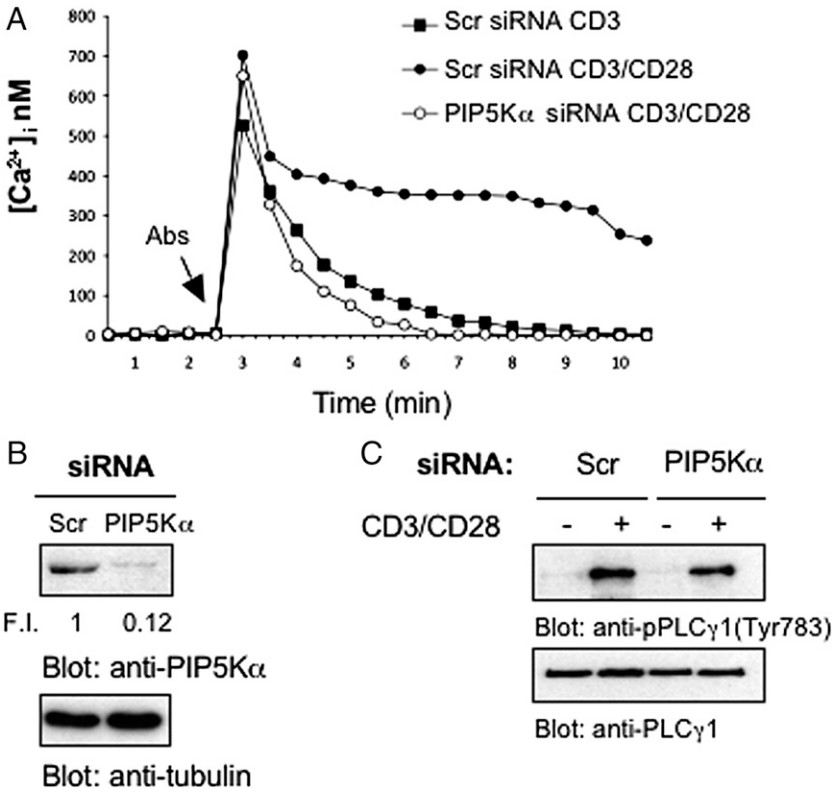

FIGURE 4. PIP5Kα knockdown impairs TCR- and CD28-induced increase of $[Ca^{2+}]_i$ in $CD4^+$ T cells. Primary $CD4^+$ T cells transfected with PIP5Kα siRNA or scrambled control siRNA (scr siRNA) for 72 h and $[Ca^{2+}]_i$, as well as PLCγ1 tyrosine phosphorylation, were analyzed after stimulation with cross-linked anti-CD3 or anti-CD3 plus anti-CD28 Abs. (**A**) Fluo-3-acetoxymethyl ester–loaded T cells (1.5×10^6) were stimulated with the indicated Abs, and changes in $[Ca^{2+}]_i$ were detected by cytofluorimetric analysis at 37°C every 24 s for 10 min. (**B**) The efficacy of PIP5Kα silencing of one representative experiment is shown. F.I. = fold inductions. (**C**) Anti-pPLCγ1 (Tyr^{783}) and anti-PLCγ1 Western blotting performed on total lysates from $CD4^+$ T cells stimulated for 5 min with cross-linked anti-CD3 plus anti-CD28 Abs. All the experiments are representative of three.

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 Ca^{2+} signaling cascade, we generated a kinase-dead mutant of PIP5Kα by substituting Asp^{310} 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 temporarily 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 PIP3 (54). PIP3 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 IA 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

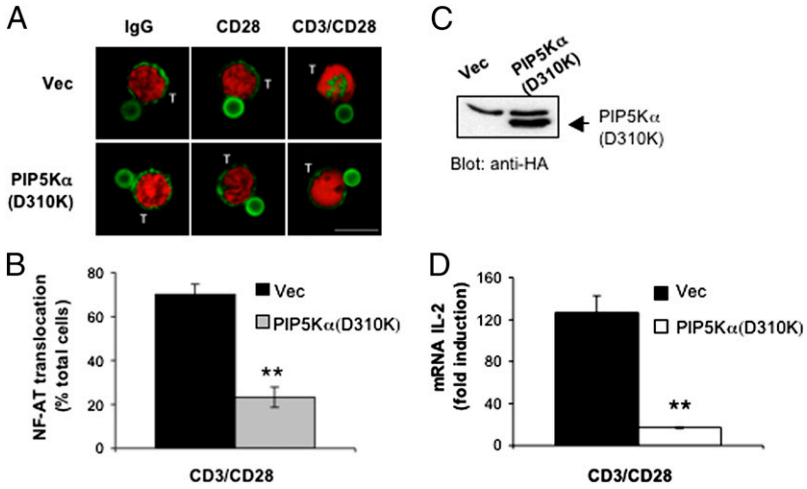

FIGURE 5. Overexpression of kinase-dead PIP5Kα (D310K) mutant impairs NF-AT nuclear translocation and IL-2 gene expression in TCR/CD28-stimulated T cells. (**A**) Primary $CD4^+$ T cells were transfected for 24 h with NF-ATc construct (1 μg) together with control empty vector (Vec) or kinase-dead HA-PIP5Kα (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.

(Fig. 1) in T lymphocytes, necessary for the activation of downstream signaling pathways.

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 Ca^{2+} from the intracellular stores. This initial wave is sustained by CD28 (46) and is necessary for Ca^{2+} influx through the gating of membrane CRAC channels, which represent the major Ca^{2+} influx pathway in T cells (60, 61). Recently, in both humans and mice, PIP5Kα isoform has been shown recruited to the plasma membrane in response to several receptors, where it provides the substrate PIP2 for PLCγ, thus inducing IP3 formation and Ca^{2+} 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 Ca^{2+} 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 PIP5K (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 Ca^{2+} 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

molecules involved in CD28-mediated activation of PIP5Kα remains our main future goal.

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Disclosures

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