Boost the immune response with
High-quality TLR Ligands
InvivoGen

The Journal of Immunology

Phosphatidylinositol 4–Phosphate 5–Kinase α and Vav1 Mutual Cooperation in CD28-Mediated Actin Remodeling and Signaling Functions

Michela Muscolini, Cristina Camperio, Nicla Porciello, Silvana Caristi, Cristina Capuano, Antonella Viola, Ricciarda Galandrini and Loretta Tuosto

J Immunol 2015; 194:1323-1333; Prepublished online 24 December 2014;
doi: 10.4049/jimmunol.1401643
http://www.jimmunol.org/content/194/3/1323

This information is current as of January 19, 2015.

Supplementary Material **http://www.jimmunol.org/content/suppl/2014/12/23/jimmunol.1401643.DCSupplemental.html**

References This article **cites 75 articles**, 43 of which you can access for free at:
http://www.jimmunol.org/content/194/3/1323.full#ref-list-1

Subscriptions Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscriptions

Permissions Submit copyright permission requests at:
http://www.aai.org/ji/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/cgi/alerts/etoc

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

Phosphatidylinositol 4–Phosphate 5–Kinase α and Vav1 Mutual Cooperation in CD28-Mediated Actin Remodeling and Signaling Functions

Michela Muscolini,* Cristina Camperio,* Nicla Porciello,* Silvana Caristi,* Cristina Capuano,† Antonella Viola,‡,§ Ricciarda Galandrini,† and Loretta Tuosto*

Phosphatidylinositol 4,5–biphosphate (PIP2) is a cell membrane phosphoinositide crucial for cell signaling and activation. Indeed, PIP2 is a pivotal source for second messenger generation and controlling the activity of several proteins regulating cytoskeleton reorganization. Despite its critical role in T cell activation, the molecular mechanisms regulating PIP2 turnover remain largely unknown. In human primary CD4$^+$ T lymphocytes, we have recently demonstrated that CD28 costimulatory receptor is crucial for regulating PIP2 turnover by allowing the recruitment and activation of the lipid kinase phosphatidylinositol 4–phosphate 5–kinase (PIP5Kα). We also identified PIP5Kα as a key modulator of CD28 costimulatory signals leading to the efficient T cell activation. In this study, we extend these data by demonstrating that PIP5Kα recruitment and activation is essential for CD28-mediated cytoskeleton rearrangement necessary for organizing a complete signaling compartment leading to downstream signaling functions. We also identified Vav1 as the linker molecule that couples the C-terminal proline-rich motif of CD28 to the recruitment and activation of PIP5Kα, which in turn cooperates with Vav1 in regulating actin polymerization and CD28 signaling functions. ***The Journal of Immunology*****, 2015, 194: 1323–1333.**

CD28 is a crucial costimulatory receptor that following engagement by its ligands, B7.1/CD80 and B7.2/CD86, provides key TCR-dependent (1, 2) and TCR-independent signals (2–6) necessary for the optimal activation of T lymphocytes. Most of CD28 autonomous and costimulatory functions rely on its unique intrinsic capability to trigger actin cytoskeleton rearrangement events, which provides the forces for the recruitment and organization of molecular signaling complexes (7). In T lymphocytes, actin cytoskeleton rearrangements are strictly regulated by signaling molecules, which couple surface receptors to the activation of specific guanine nucleotide exchange factors (GEF) for the Rho-family GTPases Rho, Rac1, and Cdc42, which link surface receptors to Wiskott–Aldrich syndrome protein (WASp) activation and Arp2/3-mediated actin nucleation (7, 8).

The dynamic and organization of actin cytoskeleton is tightly regulated by membrane phosphoinositides, which may directly interact with key actin binding proteins, thus controlling the selective localization of scaffolding molecules linking the actin cytoskeleton to the plasma membrane (9). Among the phosphoinositides, the best regulators of the actin cytoskeleton are phosphatidylinositol 3,4,5–triphosphate (PIP3) and phosphatidylinositol 4,5–biphosphate (PIP2). PIP3 is mainly generated by the activity of PI3K that phosphorylates PIP2 at the D3 position of the inositol ring, thus generating the docking site for binding the pleckstrin homology domain of important regulators of the actin cytoskeleton, such as the GEF for Rho GTPases Vav1 (10, 11). However, the optimal activation of PI3K requires the replenishment of PIP2 pools to ensure the direct regulation of actin cytoskeleton rearrangements (12).

In the majority of cell types, PIP2 is mainly generated by type I phosphatidylinositol 4–phosphate 5–kinases (PIP5K), which phosphorylate phosphatidylinositol 4–phosphate on the D5 position of the inositol ring (13). Three PIP5K isoforms (α, β, and γ) have been identified with differential subcellular localizations, thus providing both temporarily and spatially regulated distinct pools of PIP2 (14–16). Primary T cells express all three PIP5K isoforms, which are differentially enriched to the immunological synapse (IS) during T cell activation (17). PIP5Kα, for instance, is localized at the plasma membrane, where it promotes several actin-based processes (18–22). Despite much progress that has been made in elucidating the dynamic of PIP2 and PIP3 turnover in T lymphocytes, the mechanisms and surface receptors coupling PIP2 and PIP3 to cortical actin remodeling remain poorly understood.

CD28 is known to have a major role in cytoskeleton rearrangements (23–28) by triggering the tyrosine phosphorylation of Vav1 and leading to both Cdc42 and Rac1 activation (29). The connection among CD28, membrane phospholipids, Vav1, and actin-regulating proteins has long been attributed to the CD28 intrinsic ability to recruit and activate class IA PI3K and increase the cellular amount of PIP3 (30, 31). However, we have recently demonstrated that CD28 also recruits and activates PIP5Kα that ensures the PIP2 pool necessary for CD28 signaling functions (32).

*Department of Biology and Biotechnology "Charles Darwin," Pasteur Institute–Cenci Bolognetti Foundation, Sapienza University, 00185 Rome, Italy; †Department of Experimental Medicine, Sapienza University, 00185 Rome, Italy; ‡The Venetian Institute of Molecular Medicine, Padova 35129, Italy; and §Department of Biomedical Sciences, University of Padova, Padova 35121, Italy

Received for publication June 30, 2014. Accepted for publication November 17, 2014.

This work was supported by the Pasteur Institute–Cenci Bolognetti Foundation and University Research Projects (Sapienza University, Rome, Italy) and the Italian Multiple Sclerosis Foundation 2011/R/36 (Genova, Italy).

Address correspondence to Prof. Loretta Tuosto, Department of Biology and Biotechnology "Charles Darwin," Sapienza University, Via dei Sardi 70, 00185 Rome, Italy. E-mail address: loretta.tuosto@uniroma1.it

The online version of this article contains supplemental material.

Abbreviations used in this article: GEF, guanine nucleotide exchange factor; HA, hemagglutinin; IgL, Ig L chain; IS, immunological synapse; MFI, mean fluorescence intensity; PIP2, phosphatidylinositol 4,5–biphosphate; PIP3, phosphatidylinositol 3,4,5–triphosphate; PIP5Kα, phosphatidylinositol 4–phosphate 5–kinase; RRI, relative recruitment index; SEB, staphylococcal enterotoxin B; SH, Src homology; WASp, Wiskott–Aldrich syndrome protein; WT, wild-type.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/$25.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1401643

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

In this study, we extend these findings by showing that PIP5Kα is a critical regulator of CD28-mediated cytoskeleton reorganization events necessary for the activation of downstream signaling pathways. Indeed, we found that PIP5Kα cooperates with Vav1, and both are recruited to the C-terminal proline-rich motif of CD28 to regulate a common signaling pathway promoting actin polymerization in human primary $CD4^+$ T lymphocytes.

Materials and Methods

Cells, Abs, and reagents

Human primary $CD4^+$ T cells were enriched from PBMCs by negative selection using a MACS microbead sorting kit (Miltenyi Biotec, Milan, Italy) and cultured in RPMI 1640 supplemented with 5% human serum (Euroclone), L-glutamine, penicillin, and streptomycin. The purity of the sorted population was 95–99%. CD28-negative Jurkat T cell line CH7C17 (33) was maintained as described above with the addition of 400 μg/ml hygromycin B and 4 μg/ml puromycin (Sigma-Aldrich, Milan, Italy). CH7C17 cells, stable transfected with CD28 wild-type (WT), $CD28Y^{191}F$, CD28-3A (P208A, P211A, and P212A), or $CD28F^{206}F^{209}$ (Y206F and Y209F) mutants, were generated as previously described (34) and maintained as above with the addition of 2 mg/ml G418 (Sigma-Aldrich). All stable cell lines expressed comparable levels of CD28. Murine L cells Dap3, Dap3 transfected with human B7.1/CD80 (Dap/B7), HLA-DRB1*0101 (5-3.1), or 5-3.1 cotransfected with B7.1/CD80 (5-3.1/B7) to obtain CD3 and CD28 coaggregation were previously described (35, 36). The following Abs were used: goat anti-PIP5Kα (N-20), goat anti-PIP5Kα (C17), mouse anti-α tubulin, rabbit anti-Grb2 (C17), rabbit anti-Vav (H-110) and (C14), mouse anti-hemagglutin (HA) (F7), and rabbit anti-HA (Y11) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-CD28.2, mouse anti-CD3 (UCHT1), and goat anti-mouse (BD Biosciences, Milan, Italy); mouse anti-myc (9E10) (Roche, Milan, Italy); and rabbit anti-Vav, mouse anti-Rac1, mouse anti-Cdc42, sheep anti-SLP76, and GST-PAK1 (Merck Millipore, Milan, Italy). Mouse anti-FLAG (M2) Ab, PIP2, phosphatidylinositol 4–phosphate, and staphylococcal enterotoxin A were purchased from Sigma-Aldrich. Staphylococcal enterotoxin E and staphylococcal enterotoxin B (SEB) were from Toxin Technology (Sarasota, FL).

Plasmids, cell transfection, and luciferase assays

HA-tagged and FLAG-tagged PIP5Kα constructs were generated by PCR (GenBank: BC007833) from the cDNA of human primary $CD4^+$ T cells using oligonucleotides containing N-terminal NheI and C-terminal NotI restriction sites for cloning in pcDNA-HA or pcDNA-FLAG expression vectors. HA-tagged kinase-dead mutant PIP5Kα K182A mutated in the highly conserved SDDEFIIKTV sequence within the kinase core domain of human PIP5Kα (15) was generated by PCR introducing the K182A substitution into WT PIP5Kα by two-step PCR mutagenesis with the following oligonucleotides: 5′-GAGTTCATTATTGCGACAGTCCAA-CAT-3′ and 5′-ATGTTGGACTGTCGCAATAATGAACTC-3′. The PCR products were then coamplified with PIP5Kα coding sequence 5′ and 3′ primers with NheI and NotI restriction sites: 5′-TTGCTAGCGCGTCG-GCCTCCTCCGGG-3′ and 5′-TTGCGGCCGCATGGGTGAACTCTGA-CTC-3′. pEF-*Bos* expressing C-terminal myc-tagged Vav1 was previously described (37). The substitution of Vav (K335A) mutant, derived from C-terminal myc-tagged Vav1 by replacing Lys^{335} with Ala, was previously described (38). pm-Cherry and GFP constructs were generated by cloning Vav1 and PIP5Kα, either WT or mutants, in EcoR1 and BamHI restriction sites of pm-Cherry-C1 and pEGFP-N1 expression vectors (Clontech), respectively. The sequences of all PIP5Kα and Vav1 constructs were verified by DNA sequencing. pcDNA3 expressing myc-tagged Rac1 (N17) and Cdc42 (N17) constructs were kindly provided by D. Cantrell (University of Dundee, Dundee, U.K.) and A. Hall (Memorial Sloan-Kettering Cancer Center), respectively. HA-tagged human Nckβ (39) was kindly provided by W. Li (The University of Southern California, Los Angeles, CA). FLAG-tagged SLP-76 WT was kindly provided by G.A. Koretzky (Weill Cornell Medical College, New York, NY).

The NF-κB luciferase gene under the control of six thymidine kinase NF-κB sites was kindly provided by J.F. Peyron (Faculté de Médicine Pasteur, Nice, France). The NFAT luciferase reporter construct containing the luciferase gene under the control of the human IL-2 promoter NFAT binding site was kindly provided by C. Baldari (University of Siena, Siena, Italy).

Primary $CD4^+$ T cells, resuspended in 100 μl Nucleofector solution (Amaxa Biosystems), were electroporated with 1 μg indicated expression vector using the V-024 program of the Nucleofector.

For luciferase assays, 10^7 cells were electroporated (at 260 V, 960 μF) in 0.5 ml RPMI 1640 supplemented with 20% FCS with 2 μg NF-κB luciferase or 10 μg NFAT luciferase together with 5 μg pEGFP and 20 μg each indicated expression vector, keeping the total amount of DNA constant (40 μg) with empty vector. Twenty-four hours after transfection, cells were stimulated with Dap3 or Dap/B7 or 5-3.1 or 5-3.1/B7 cells prepulsed with SEB (1 μg/ml) at 37°C for 6 h. Luciferase activity was measured according to the manufacturer's instruction (Promega). Luciferase activity determined in triplicates was expressed as arbitrary luciferase units after normalization to GFP values.

Cell viability was determined by quantifying the ability of cells to incorporate trypan blue and expressed as percent viability.

Cell stimulation and immunoblotting

Primary $CD4^+$ T cells and Jurkat 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-A (GE Healthcare) or protein G Sepharose (Sigma-Aldrich) and then immunoprecipitated for 2 h with the indicated Abs preadsorbed on Protein-G or -A Sepharose beads. 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 goat anti-mouse (Amersham) or HRP-labeled donkey anti-goat Abs (Santa Cruz Biotechnology), developed with the ECL's detection system (GE Healthcare). For anti-Grb2 Western blotting, HRP-labeled protein-A (GE Healthcare) was used to reduce Ig L chain (IgL) binding.

PIP5Kα kinase assay and measurement of PIP2 levels

Primary $CD4^+$ T cells (20×10^6) were stimulated for 5 min with adherent Dap3 or Dap3/B7 cells and 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. CH7C17 CD28WT Jurkat cells were transfected with HA-PIP5Kα WT or HA-PIP5Kα (K182A) mutant. Cells were 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; endogenous or exogenous PIP5Kα was immunoprecipitated and incubated with phosphatidylinositol 4–phosphate as previously described (40). Briefly, phosphatidylinositol 4–phosphate was resuspended in assay buffer (30 mM HEPES [pH 7.4], 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, and 0.05% Nonidet P-40) and sonicated. Beads containing PIP5Kα were incubated with 50 μl assay buffer containing phosphatidylinositol 4–phosphate, 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 phosphatidylinositol 4–phosphate 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).

Confocal microscopy

A total of 15×10^3 murine L cells was adhered on cover glasses (12 mm) overnight at 37°C. T cells or CH7C17 cells expressing CD28 WT or CD28 mutants were transfected for 24 h with 1 μg or 20 μg, respectively, of the indicated expression vectors, 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. F-actin was stained by using phalloidin-633 (Molecular Probes). Confocal observations were performed using a Leica DMIRE apparatus (Leica Microsystems, Heidelberg, Germany) equipped with an argon-krypton laser and double-dichroic splitters (488/568 nm). Image acquisition and processing were conducted by using the Leica confocal software (Leica Microsystems). Images were analyzed with the Adobe Photoshop program (Adobe Systems). The relative recruitment index (RRI) was calculated as previously described by the 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 CD28 internalization

CD28-negative CH7C17 cells were transfected for 24 h with 10 μg PEF-*Bos*-CD28 WT construct alone or together 20 μg HA-PIP5Kα WT or HA-PIP5Kα (K182A) mutant. Cells were incubated with anti-CD28.2 (10 μg/ml) or isotype control mAb at 4°C to prevent receptor internalization, washed, and either maintained at 4°C or shifted to 37°C for different times to allow receptor internalization. At the end of incubation, cells were returned to 4°C to

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

stop receptor internalization, incubated with Alexa 488–conjugated secondary Ab, and analyzed by flow cytometry (FACSCalibur; BD Biosciences, Milan, Italy). The percentage of CD28 remaining on the cell membrane was calculated from the MFI values as: 100 × (MFI [37°C sample] − MFI [37°C isotype control]/MFI [4°C sample] − MFI [4°C isotype control]).

Measurement of conjugate formation

Conjugate formation was measured as previously described (25). Briefly, CH7C17 Jurkat cells expressing CD28 WT were transfected with GFP-PIP5Kα WT or GFP-PIP5Kα K182A constructs, and transfectants (3.5×10^6) were incubated for 5 min at 37°C with Dap/B7 (1.2×10^6) in a final volume of 70 μl RPMI 1640, then diluted in 500 μl RPMI 1640, and analyzed by FACS. Conjugates were identified on a total of 10^5 GFP-positive events by gating for side scatter and forward light scatter (41) and expressed as mean percentage ± SD of triplicate samples.

Real-time PCR

Total RNA was extracted using the RNeasy MicroKit (Qiagen) from 5×10^5 cells and 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 threshold cycle method.

Statistical analysis

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

Results

PIP5Kα lipid kinase activity regulates CD28-mediated actin cytoskeleton reorganization events required for CD28-dependent signals

We have recently demonstrated that, in the absence of TCR engagement, CD28 stimulation by either B7.1/CD80 or agonistic Abs recruits and activates PIP5Kα in human primary $CD4^+$ T

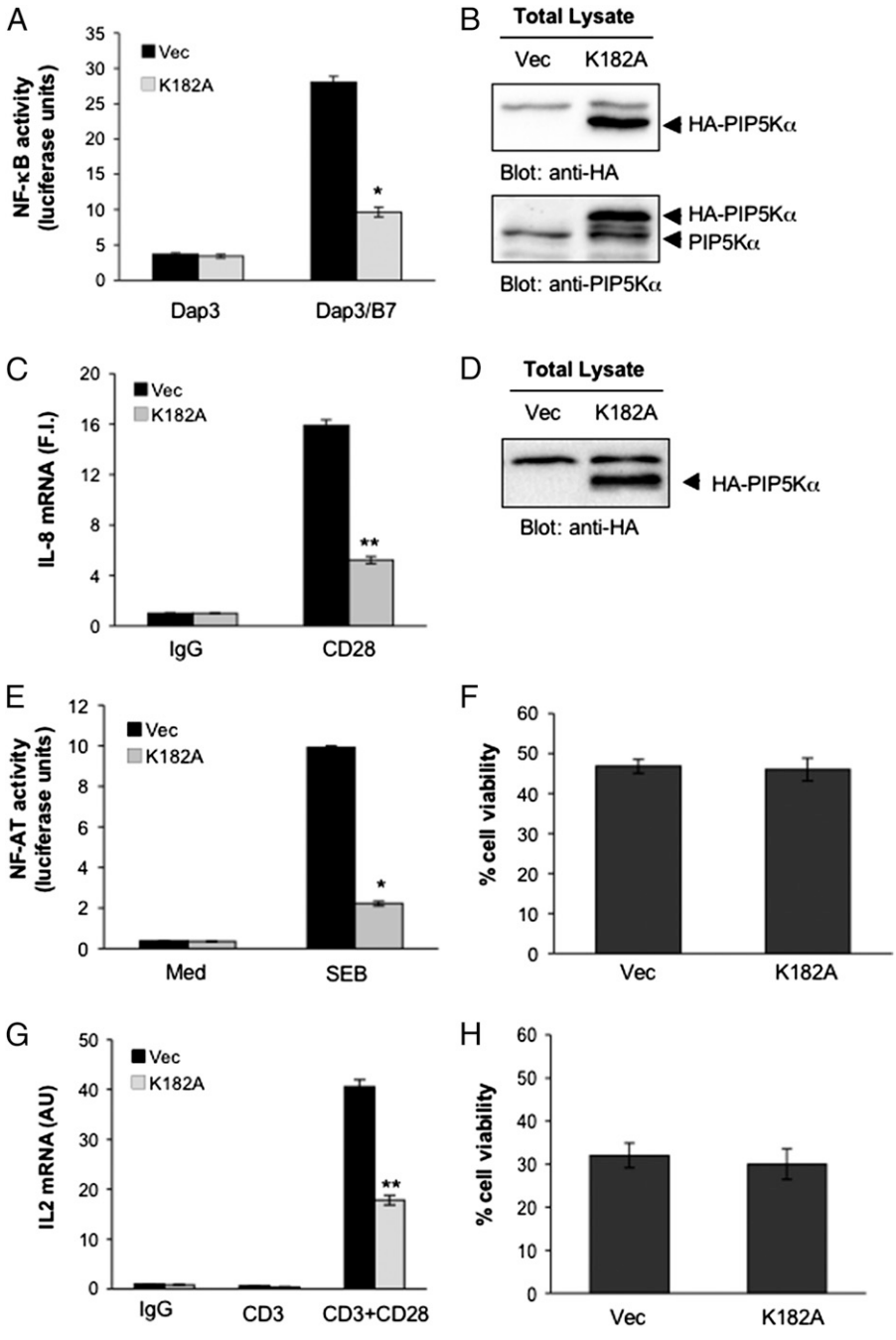

FIGURE 1. PIP5Kα kinase activity is required for CD28-induced functions. (**A**) NF-κB luciferase activity of Jurkat cells expressing CD28 WT transfected with empty vector or HA-PIP5Kα K182A mutant and stimulated with B7-negative (Dap3) or Dap/B7 cells. (**E**) NFAT luciferase activity of CD28 WT cells transfected with empty vector or HA-PIP5Kα K182A mutant and stimulated with 5-3.1/B7 cells prepulsed or not (Med) with SEB (1 μg/ml). The results are expressed as the mean of luciferase units ± SD after normalization to GFP values. The data are representative of three independent experiments. *$p < 0.001$ calculated by Student *t* test, compared with stimulated cells transfected with Vec. Real-time PCR was used to measure IL-8 mRNA (**C**) or IL-2 mRNA (**G**) levels in primary $CD4^+$ T cells transfected with empty vector (Vec) or HA-PIP5Kα (K182A) mutant and stimulated for 6 h with control IgG or anti-CD28 Abs (C) or anti-CD3 or anti-CD3 plus anti-CD28 Abs (G). Data are expressed as fold inductions (F.I.) over the basal level of cells stimulated with control IgG (C) or arbitrary units (AU) (G). Bars show the mean ± SD of three independent experiments. **$p < 0.01$ calculated by Student *t* test, compared with anti-CD28 (C) or anti-CD3 plus anti-CD28–stimulated (G) cells transfected with Vec. Anti-HA and anti-PIP5Kα Western blottings were performed on total lysates from CD28 WT cells (**B**) or primary $CD4^+$ T cells (**D**) transfected with Vec or HA-PIP5Kα K182A. The positions of endogenous PIP5Kα (B) or HA-PIP5Kα K182A (B and D) are indicated on the *right*. Cell viability was determined by quantifying the ability of transfected CD28 WT cells (**F**) or primary $CD4^+$ T cells (**H**) to incorporate trypan blue and expressed as the mean percentage ± SD of three independent experiments.

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

lymphocytes (Supplemental Fig. 1A) and that silencing of PIP5Kα impairs CD28-mediated signaling functions (32). The lipid-kinase activity of PIP5Kα was crucial for the regulation of both CD28 unique signals or CD28/TCR costimulatory signals, as demonstrated by the inhibitory effects exerted by overexpressing a lipid-kinase-dead mutant of PIP5Kα (K182A) (15, 42) (Supplemental Fig. 1B) in both primary $CD4^+$ T cells and Jurkat cells (Fig. 1). Indeed, the overexpression of PIP5Kα (K182A) mutant strongly impaired CD28 autonomous signaling regulating NF-κB transcriptional activation in both CH7C17 cells expressing CD28 WT (Fig. 1A) and Jurkat cells (Supplemental Fig. 1E). Consistent with the role of RelA/NF-κB in regulating IL-8 gene transcription in $CD4^+$ T cells (3, 6), the overexpression of PIP5Kα (K182A) significantly inhibited IL-8 transcription (65%) induced by CD28 individual ligation (Fig. 1C). PIP5Kα (K182A) overexpression also impaired NFAT transcriptional activity (Fig. 1E) and IL-2 gene expression induced by CD28 costimulation (Fig. 1G). Any effects on cell viability were observed in neither CD28 WT Jurkat cells (Fig. 1F) nor primary $CD4^+$ T cells (Fig. 1H) following expression of PIP5Kα K182A mutant.

Most of the CD28-mediated signaling functions rely on its intrinsic ability to regulate the remodeling of actin cytoskeleton necessary for the initiation of both autonomous and TCR costimulatory signaling (26, 34, 43). Thus, we explored the role of PIP5Kα in CD28-induced actin remodeling. Confocal microscopy analyses revealed that CD28 stimulation by B7 induced PIP5Kα recruitment as well as the polarization and accumulation of F-actin at the T–APC interface (Fig. 2A). In contrast, K182A mutation strongly impaired PIP5Kα polarization at the T–APC contact zone following CD28 engagement by B7. Interestingly enough, the overexpression of PIP5Kα (K182A) impaired the polarization and accumulation of F-actin in B7-stimulated cells (Fig. 2A), without affecting conjugate formation (Fig. 2B), thus evidencing a crucial role of PIP5Kα in regulating CD28-induced actin remodeling.

Because actin-based processes are needed for CD28 internalization (30, 44) and PIP5Kα has been also implicated in receptor-mediated endocytosis (20, 21), we investigated whether PIP5Kα might play a role in CD28 internalization. To this end, CD28-negative CH7C17 Jurkat cells were cotransfected with CD28 WT expression vector together with empty vector or HA-PIP5Kα WT or K182A mutant. As shown in Fig. 2C, CD28.2 mAb induced a marked downmodulation of CD28 of 40–50% by 1 h up to 70% by 8 h. No significant differences on the rate of CD28 downregulation were observed in cells expressing PIP5Kα WT or PIP5Kα (K182A) compared with the empty vector.

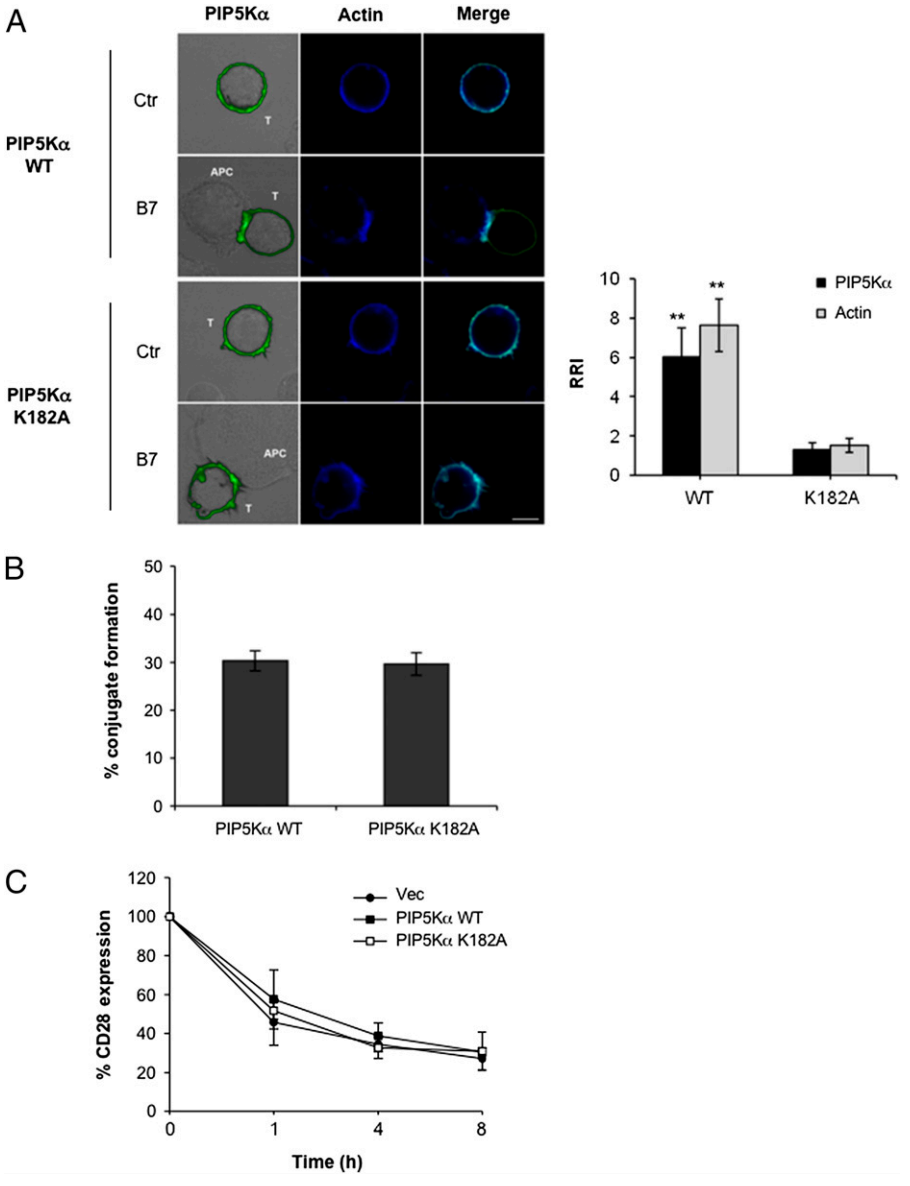

FIGURE 2. PIP5Kα kinase-dead mutant impairs CD28-mediated actin cytoskeleton reorganization without affecting CD28 downregulation. (**A**) CD28 WT cells were transfected with GFP-PIP5Kα WT or K182A mutant constructs (10 μg) for 24 h and then stimulated for 15 min in the absence (Ctr) or presence of Dap/B7 cells (B7). After fixing and permeabilization, F-actin (blue) was stained with 633-conjugated phalloidin and analyzed by confocal microscopy. Scale bar, 10 μm. The RRI was calculated as described in *Materials and Methods* and represents the mean ± SD of 15 conjugates analyzed in each group. $^{**}p < 0.01$ calculated by Student *t* test compared with controls. More than 70% of T–Dap/B7 conjugates showed PIP5Kα WT RRI >5. The mean percentage ± SD of PIP5Kα WT and PIP5Kα K182A localization in the T–Dap/B7 contact zone was 84 ± 4.8 and 36 ± 3, respectively. The results are representative of three independent experiments. (**B**) CD28 WT cells were transfected with GFP-PIP5Kα WT or K182A mutant for 24 h and then stimulated for 5 min with Dap/B7 cells. Conjugate formation was measured by FACS and expressed as the mean percentage ± SD of three independent experiments performed in triplicates. (**C**) CH7C17 Jurkat cells were cotransfected with CD28 WT and with empty vector (Vec) or HA-PIP5Kα WT or K182A mutant constructs (20 μg) for 24 h, and surface CD28 downregulation was measured by FACS analysis as described in *Materials and Methods*. The results express the percentage of CD28 expression on the surface. Data represent the mean ± SD of three independent experiments.

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

Altogether, these data indicate that the kinase activity of PIP5Kα controls CD28-mediated actin remodeling processes needed for CD28 signaling functions but not for receptor endocytosis.

PIP5Kα/Vav1 complexes are recruited to the T–APC interface and regulate Vav1-dependent signaling functions in response to CD28 stimulation

In T lymphocytes, Vav1 is a critical GEF for Rac1 and Cdc42 GTPases (45) and required for CD28-dependent signals and actin nucleation (1, 2, 46, 47). We analyzed if Vav1 and PIP5Kα were corecruited and accumulated at the T–APC interface following CD28 engagement. Primary $CD4^+$ T cells were transfected with GFP-PIP5Kα WT together with pmCherry-Vav1 WT constructs and then stimulated for 15 min with B7.1/CD80-positive ($B7^+$) or B7.1/CD80-negative ($B7^-$) cells. Confocal analyses revealed that Vav1 was efficiently recruited and polarized with PIP5Kα at the T–APC contact zone (Fig. 3A). To assess the role of PIP5Kα kinase activity in regulating Vav1-dependent signals downstream

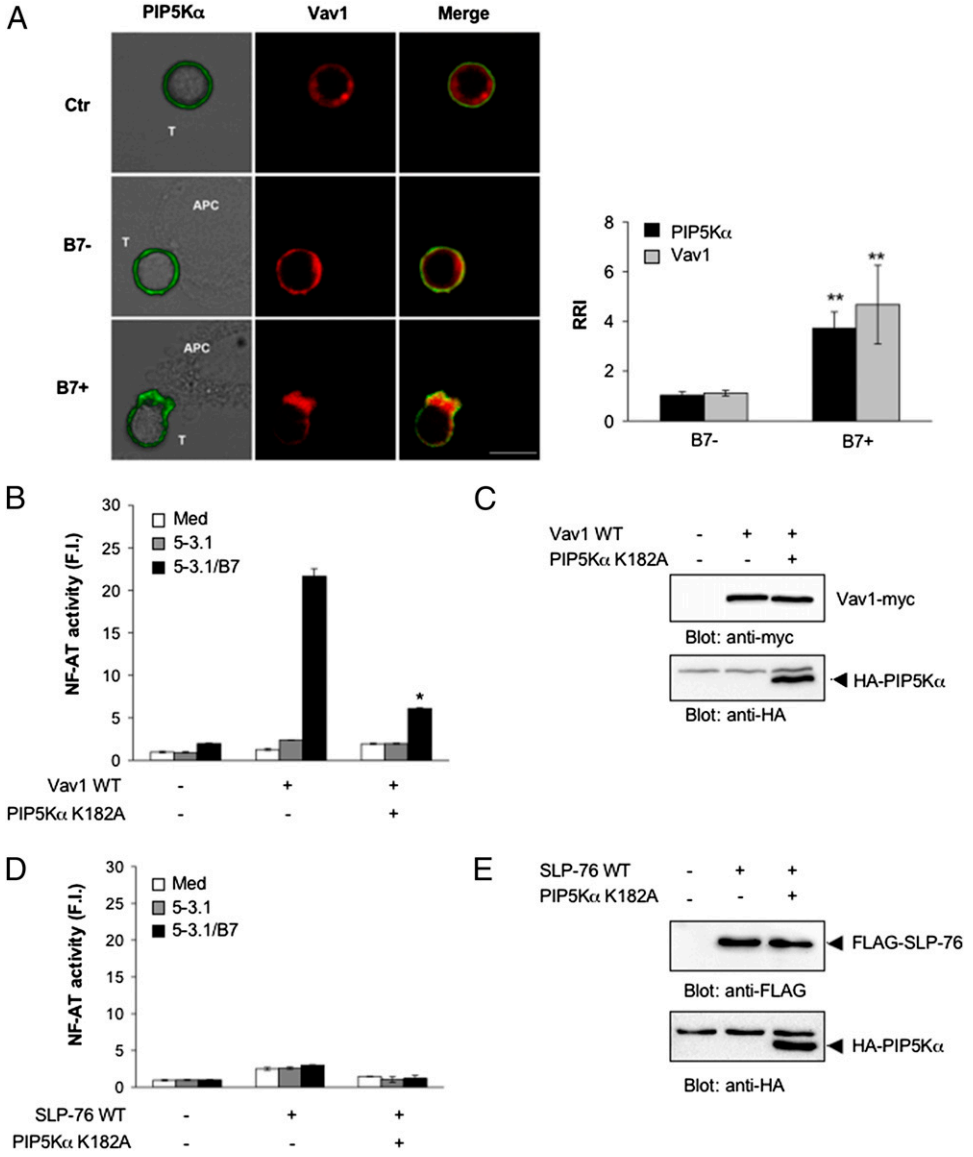

FIGURE 3. PIP5Kα kinase-dead mutant impairs Vav1-dependent NFAT activation in CD28 stimulated cells. (**A**) Primary $CD4^+$ T cells were transfected with 1 μg GFP-PIP5Kα WT together with 1 μg pCherry-Vav1 constructs and then stimulated for 15 min in the absence (Ctr) or presence of adherent murine B7-negative ($B7^-$) or B7-positive ($B7^+$) cells. After fixing, cells were analyzed by confocal microscopy. Scale bar, 10 μm. The RRI of Vav1 (red) and PIP5Kα (green) was calculated and represents the mean ± SD of 15 conjugates analyzed in each group. $^{**}p < 0.001$ calculated by Student *t* test compared with controls. The mean percentage ± SD of Vav1 and PIP5Kα localization in the T–APC/B7 contact zone was 86 ± 4 and 83 ± 5, respectively. The results are representative of three independent experiments. (**B**) NFAT luciferase activity of CD28 WT cells transfected with empty vector (−) or Vav1 WT alone or in combination with HA-PIP5Kα K182A mutant and stimulated in the absence (Med) or presence of B7-negative (5-3.1) or 5-3.1/B7 cells. Data are expressed as fold inductions (F.I.) over the basal level of cells transfected with empty vector (−) and unstimulated (Med), after normalization to GFP values. Bars show the mean ± SD of one experiment representative of three. $^{*}p < 0.001$ calculated by Student *t* test, compared with stimulated cells transfected with Vav1 WT alone. (**C**) The expressions of myc-Vav1 (*top panel*) and HA-PIP5Kα K182A (*bottom panel*) were analyzed by Western blotting. The position of HA-PIP5Kα K182A is indicated on the *right*. The *upper band* is a nonspecific band recognized by the anti-HA Ab. All data are representative of three independent experiments. (**D**) NFAT luciferase activity of CD28 WT cells transfected with empty vector (−) or FLAG-SLP-76 WT alone or in combination with HA-PIP5Kα K182A mutant and stimulated in the absence (Med) or presence of B7-negative (5-3.1) or 5-3.1/B7 cells. Data are expressed as F.I. over the basal level of cells transfected with empty vector (−) and unstimulated (Med), after normalization to GFP values. Bars show the mean ± SD. (**E**) The expressions of FLAG–SLP-76 (*top panel*) and HA-PIP5Kα K182A (*bottom panel*) were analyzed by Western blotting.

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

of CD28, we looked at NFAT activation, an event that we have previously demonstrated to depend on both Vav1 (37) and PIP5Kα (32). As already described (37), overexpression of Vav1 strongly activated NFAT-dependent transcription induced by CD28 engagement in the absence of TCR engagement. The expression of PIP5Kα (K182A) dominant-negative mutant strongly impaired Vav1-mediated NFAT activation in CD28-stimulated cells (Fig. 3B), without affecting cell viability (data not shown). On the contrary, the overexpression of SLP-76, a known Vav1 binding partner (48, 49), did not activate NFAT following CD28 engagement, and, consequently, PIP5Kα K182A did not exert any significant effect (Fig. 3D).

These findings suggest a tight connection between PIP5Kα and Vav1 in CD28 signaling functions.

Reciprocal interdependence of Vav1 and PIP5Kα activities in regulating CD28 actin reorganization

Our data on the corecruitment of Vav1 and PIP5Kα, together with the ability of PIP5Kα to regulate actin polymerization in response to CD28, prompted us to investigate the specific contribution of each molecule in their reciprocal recruitment. More than 75% of conjugates expressing PIP5Kα WT exhibited a strong polarization and accumulation of Vav1 at the T–APC contact site. On the contrary, the percentage of conjugates with polarized Vav1 was strongly reduced (12%) in PIP5Kα-transfected (K182A) cells (Fig. 4A). Interestingly, when we looked at the role of Vav1 GEF activity on PIP5Kα recruitment, we found similar results. Indeed, the overexpression of Vav1 (K335A), a mutation that impairs Vav1 GEF activity and its ability to induce actin rearrangement (50), reduced the percentage of conjugates (6%) exhibiting PIP5Kα recruitment at the T–APC interface (Fig. 4B). No differences in the total number of conjugates were observed in cells expressing Vav1 (K335A) or PIP5Kα (K182A) mutants compared with the WT (not shown).

Altogether, these data evidence a reciprocal interdependence of Vav1 and PIP5Kα activities in regulating CD28 actin rearrangements functions.

The C-terminal proline-rich motif of CD28 is essential for Vav1 and PIP5Kα recruitment and CD28 costimulatory functions

CD28 short cytoplasmic tail has no enzymatic activity but contains several motifs crucial for the activation of downstream signaling cascade. CD28 contains an N-terminal YMNM motif that following phosphorylation binds the p85 subunit of PI3K (51–53) and Grb2 (54, 55), an N-terminal proline rich domain that binds the Src homology (SH) 3 domain of Itk (56), and a C-terminal proline rich motif (YQPYAPP) that binds the SH3 domain of Lck (57) and is critical for the recruitment of the actin-binding protein filamin A (26, 34). Because we have recently found that the C-terminal proline-rich motif of CD28 is involved in the recruitment of PIP5Kα (32), we verified if the same domain also regulated Vav1 polarization. To this aim, we used Jurkat cells expressing CD28 WT or CD28 mutants in the YNMN motif (CD28 $Y^{191}F$) or in the C-terminal YQPYAPP motif (CD28-3A). Confocal microscopy analyses evidenced that CD28 $Y^{191}F$ mutant did not affect the localization and recruitment of neither Vav1 nor PIP5Kα. On the contrary, CD28-3A mutant failed to recruit both PIP5Kα and Vav1 at the T–APC interface (Fig. 5A) and to induce actin rearrangement (Supplemental Fig. 4A) without affecting CD28 downregulation (Supplemental Fig. 4B). Consistent with the key role of PIP5Kα and Vav1 in the regulation of CD28 signaling functions, both IL-8 (Fig. 5B) and IL-2 (Fig. 5C) gene expressions were

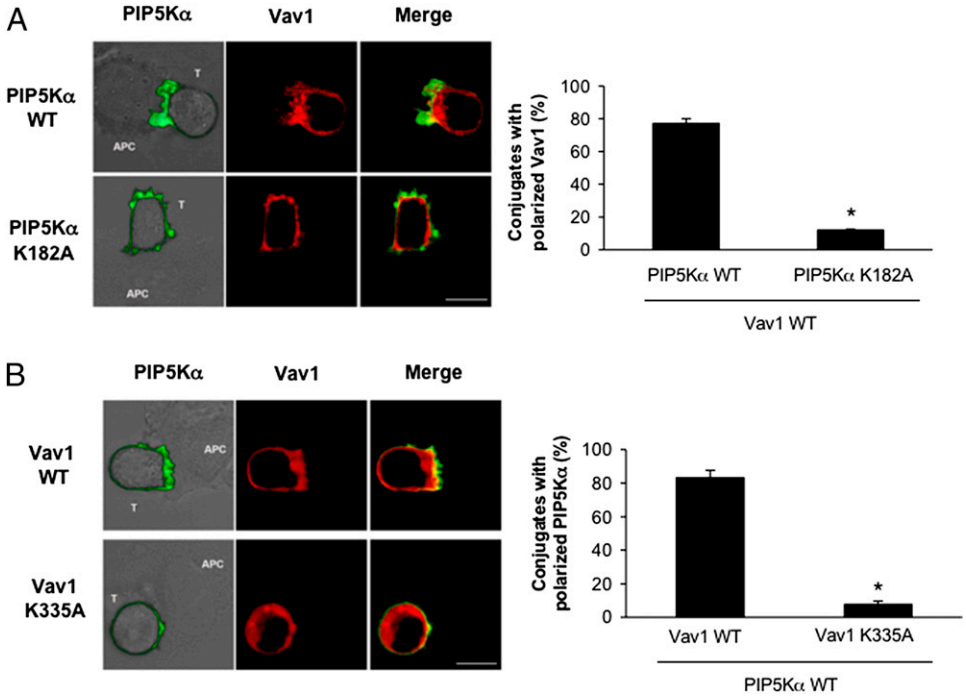

FIGURE 4. PIP5Kα and Vav1 activities are required for their reciprocal recruitment to CD28. CD28WT cells were transfected with pm-Cherry Vav1 vector together with GFP-PIP5Kα WT or K182A mutant constructs (**A**) or with GFP-PIP5Kα WT together with pm-Cherry Vav1 WT or Vav1 K335A mutant (**B**); after 24 h, cells were stimulated for 15 min with adherent Dap/B7 cells. After fixing, cells were analyzed by confocal microscopy. Scale bar, 10 μm. The frequency of conjugates (total 30) showing significant Vav1 (A, *right panel*) or PIP5Kα WT (B, *right panel*) polarizations (RRI >2.5) were calculated, and data represent the mean ± SD of three independent experiments. The localization of each protein in the T–Dap/B7 contact zone was quantified, and the mean percentages ± SD were as following; in PIP5Kα WT-expressing cells, Vav1 WT was 75 ± 8, and PIP5Kα WT was 78 ± 7; in PIP5Kα K182A-expressing cells, Vav1 WT was 30 ± 8, and PIP5Kα K182A was 35 ± 7; in Vav1 K335A-expressing cells, PIP5Kα WT was 36 ± 6, and Vav1 K335A was 30 ± 7. *$p < 0.001$ calculated by Student t test compared with controls.

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

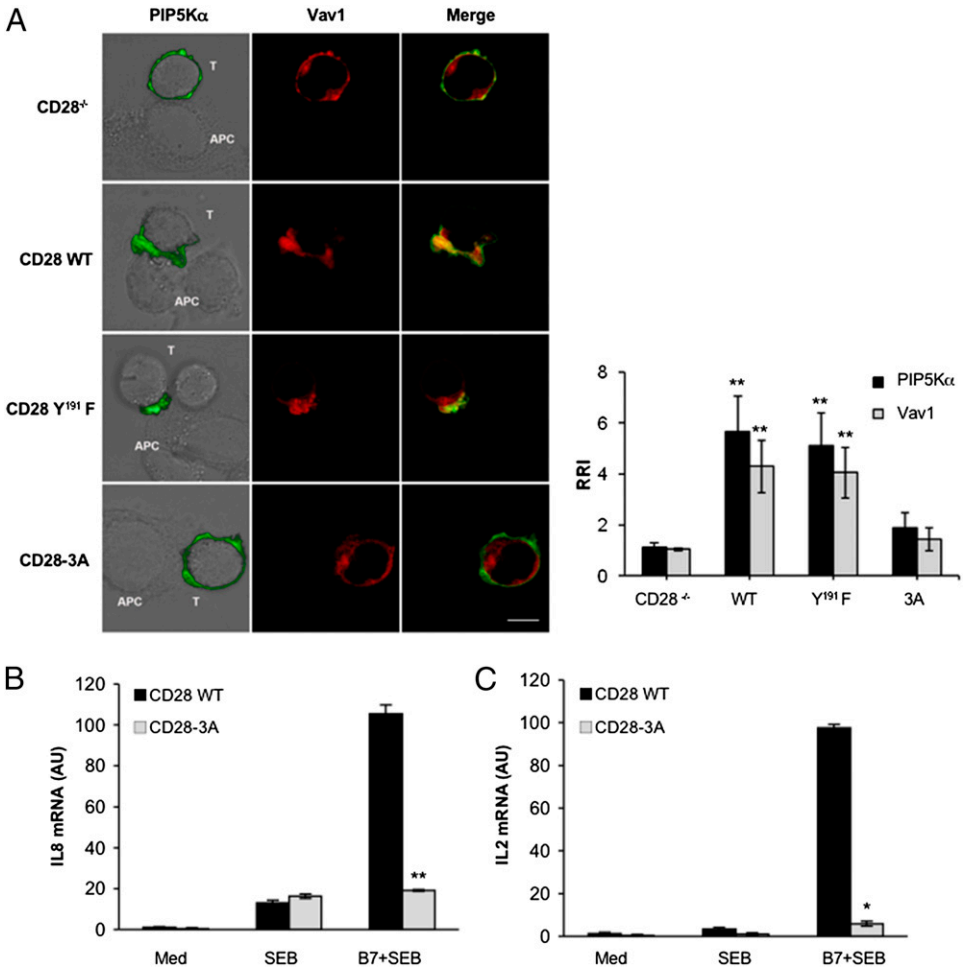

FIGURE 5. The C-terminal proline rich motif of CD28 is essential for Vav1 and PIP5Kα recruitment and biological functions. (**A**) CD28-negative CH7C17 cells ($CD28^{-/-}$) or CD28 WT or CD28 $Y^{191}F$ or CD28-3A mutants were transfected with 20 μg of GFP-PIP5Kα together with pm-Cherry-Vav1 constructs and then stimulated for 15 min with adherent Dap/B7 cells. After fixing, cells were analyzed by confocal microscopy. Scale bar, 10 μm. The RRI of Vav1 (red) and PIP5Kα (green) was calculated and represents the mean ± SD of 15 conjugates analyzed in each group. $^{**}p < 0.01$ calculated by Student *t* test compared with controls. Real-time PCR was used to measure IL-8 (**B**) and IL-2 (**C**) mRNA levels in CD28 WT or CD28-3A cells stimulated for 6 h with 5-3.1 cells or 5-3.1/B7 cells pulsed or not (Med) with 1 μg/ml SEB. Data are expressed as arbitrary units (AU). Bars show the mean ± SD of three independent experiments. $^{*}p < 0.001$, $^{**}p < 0.01$, respectively, calculated by Student *t* test, compared with SEB-pulsed 5-3.1/B7 CD28 WT cells.

strongly impaired by mutation in the C-terminal proline-rich domain of CD28, and the overexpression of PIP5Kα WT did not reverse the inhibition mediated by CD28-3A mutant (data not shown).

The mechanism suggested for Vav1 recruitment to CD28 involves Grb2 (58), which has been found to interact with both Vav1 (59, 60) and the YMNM as well as the C-terminal proline-rich motif of CD28 (54, 61). To elucidate the dynamic of Vav1 and PIP5Kα recruitment to CD28, primary $CD4^+$ T cells were stimulated or not with B7-expressing APC and the association of Vav1 or PIP5Kα with CD28 was analyzed. Consistent with previous reported data (58), Vav1 coprecipitated with CD28 following stimulation (Fig. 6A, *top panel*). Any direct association between CD28 and PIP5Kα was found (Fig. 6A, *middle panel*). Moreover, when we looked at Grb2, we did not observe any association with CD28 in unstimulated or stimulated human primary $CD4^+$ T cells (Fig. 6B, *top panel*). Similar results were obtained in Jurkat cells (Supplemental Fig. 2A). Consistent with our previously published data (3), the p85 adaptor subunit of PI3K efficiently bound to CD28 in stimulated primary $CD4^+$ T cells (Fig. 6B, *middle left panel*), and Grb2 was found constitutively associated with SLP76 in unstimulated cells (Fig. 6B, *top right panel*). No association between Vav1 and Grb2 or PIP5Kα and Grb2 was detected in primary $CD4^+$ T cells (Fig. 6C).

We next investigated which domain of CD28 was involved in the association with Vav1. Jurkat cells expressing CD28 WT or CD28 $Y^{191}F$ or two mutants within the C-terminal proline-rich motif, CD28-3A or CD28 $F^{206}F^{209}$, were transfected with myc-Vav1 WT, and CD28/Vav1 association was analyzed after stimulation with Dap/B7 cells. As expected, the mutation of YMNM motif impaired the recruitment of the p85 regulatory subunit of PI3K (Supplemental Fig. 2B) without affecting Vav1 binding to CD28 (Fig. 6D). On the contrary, CD28-3A and CD28 $F^{206}F^{209}$ mutants in the C-terminal proline-rich motif exhibited a strong reduction of Vav1 binding (Fig. 6D).

Altogether, these data suggest that Vav1 may mediate CD28/PIP5Kα association in a Grb2-independent manner by binding either directly or indirectly to phosphotyrosine residues within the C-terminal proline-rich motif of CD28.

PIP5Kα associates with Vav1 in a trimolecular complex containing Nck

There is extensive evidence that Rho family GTPases recruit and activate PIP5Ks. Rac1 and Cdc42 can interact with all PIP5K isoforms in a GTP-independent manner allowing PIP5Ks recruitment to the plasma membrane (18, 19, 42, 62). In contrast to that observed in other cell types, no association of endogenous PIP5Kα with Rac1 or Cdc42 was observed in primary $CD4^+$ T lymphocytes neither unstimulated nor activated with adherent B7-expressing cells (Fig. 7A, *bottom panel*). On the contrary, we found that PIP5Kα constitutively associated with Vav1 in unstimulated primary T cells, and the association did not change following CD28 stimulation (Fig. 7A, *top panel*). Efficient binding of activated Cdc42 and Rac1 with PAK-1 was observed in primary $CD4^+$ T lymphocytes (Fig. 7B). Similar results were obtained when PIP5Kα was coexpressed with Rac1 (Fig. 7C), Cdc42 (Fig. 7D), and Vav1 (Fig. 7E) in Jurkat cells. Consistent with previously published data (63), we also found that Vav1 associated

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

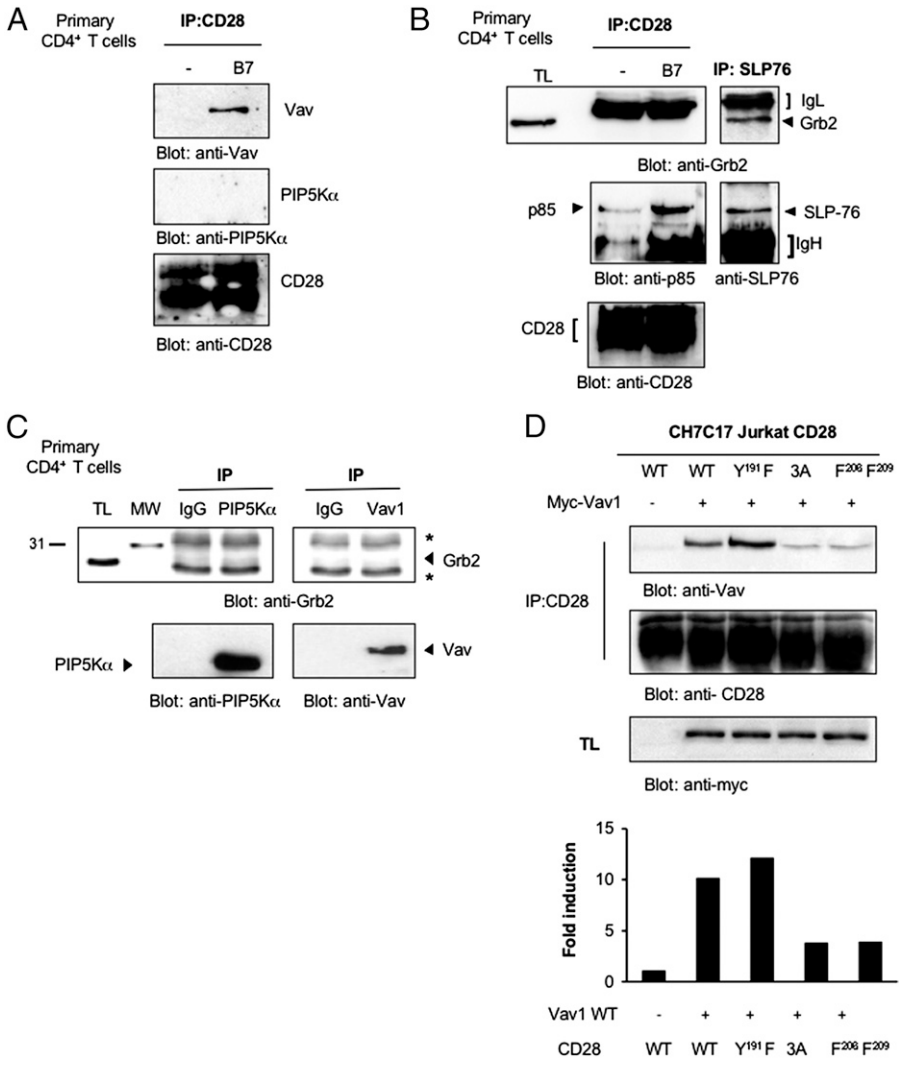

FIGURE 6. Vav1 binds to the CD28 C-terminal proline-rich motif independently of Grb2. (**A** and **B**) Primary $CD4^+$ T cells were stimulated for 5 min with B7-negative (−) or Dap/B7 cells, and anti-CD28 immunoprecipitation (IP) was performed on cellular extracts. Anti-Vav, anti-PIP5Kα, anti-p85 PI3K, anti-CD28, and anti-Grb2 Western blottings were performed on anti-CD28 IP or total lysates (TL). In unstimulated primary $CD4^+$ T cells, anti-Grb2 Western blotting was also performed on SLP-76 IP (B). The position of immunoprecipitated proteins as well as of IgL or Ig H chain (IgH) are indicated. (**C**) Anti-Grb2 (*top panel*) Western blotting of TL or isotype-matched control IgG or anti-PIP5Kα or anti-Vav IP from unstimulated primary $CD4^+$ T cells (*top panel*). Each IP was analyzed for PIP5Kα and Vav content (*bottom panel*). All data are representative of four independent experiments. (**D**) CD28 WT, $Y^{191}F$, 3A, and $F^{206}F^{209}$ cells were transfected with myc-Vav1 where indicated, stimulated with Dap/B7 cells, and the association of Vav1 with CD28 was analyzed by Western blotting in anti-CD28 IP (*top panel*). The levels of CD28 (*top middle panel*) or myc-Vav1 expression (*bottom middle panel*) were also analyzed. Fold induction (*bottom panel*) of Vav1 in anti-CD28 IP was quantified and normalized to both CD28 and myc-Vav1 levels. The results are representative of three independent experiments. *IgL chains.

with Nckβ (Fig. 7E, 7F), a critical adaptor that cooperates with PIP5Ks in promoting N-WASp localization and actin polymerization (64). No direct association between PIP5Kα and Nck was observed (Fig. 7F). The association of Vav1 with PIP5Kα was not affected by either K335A or K182A mutations (Fig. 7G).

Based on these results, we propose that, in human T lymphocytes, Vav1 binds to the C-terminal proline-rich motif of CD28 after its engagement, thus favoring the initial recruitment of both PIP5Kα and Nck. PIP5Kα synergizes with PI3K, generating the high local concentration of PIP2 and PIP3, which in turn stabilizes the membrane localization of Vav1 and Nck/WASp/Arp2-3 complexes needed to promote actin polymerization and activation of downstream signaling pathways (Supplemental Fig. 3).

Discussion

T cell activation is accompanied by a dynamic reorganization of actin cytoskeleton that allows clustering of TCR and costimulatory receptors toward the T–APC interface. CD28 costimulation is crucial for the cytoskeleton rearrangement events required for the relocalization of receptors, lipid rafts, and signaling complexes at the IS (23–27). Interestingly, CD28 regulates the remodeling of the actin cytoskeleton independently of TCR (29, 43), thus delivering a unique signal necessary for both the initiation of TCR signaling as well as for CD28 autonomous functions (47, 65). Despite much progress that has been made in elucidating the mechanisms involved in CD28-mediated cytoskeleton reorganization, the upstream molecules coupling CD28 triggering to cortical actin remodeling remain poor understood. In this study, we demonstrate that PIP5Kα and Vav1 are critical mediators of CD28-dependent actin rearrangement necessary for its biological activities.

PIP2 are pivotal for the remodeling of the actin cytoskeleton by binding and activating several actin regulating proteins and affecting various cellular processes, such as endocytosis, exocytosis, and membrane ruffles (9). In different cell types and in response to several receptors, PIP5Kα promotes actin assembly and membrane ruffles (18, 19), receptor-mediated phagocytosis, and endocytosis (20, 21) as well as lytic granule exocytosis and retrieval (22, 66). In T lymphocytes, the spatiotemporal analysis of both PIP2 distribution and turnover evidenced that PIP2 concentrates at the IS very early during Ag recognition (67) and that PIP2 synthesis occurs at the T–APC interface, where PIP5Kα accumulates in a sustained fashion (17). In this study, we evidence that CD28 is the main regulator of PIP5Kα recruitment to the T–APC interface and that PIP5Kα activity is essential for CD28-mediated actin polymerization (Fig. 2A). Indeed, kinase-dead PIP5Kα (K182A) retains its localization at the plasma membrane (62), but fails to polarize to the T–APC contact zone. Furthermore, we also found that PIP5Kα activity is dispensable for CD28 endocytosis (Fig. 2C). These data are consistent with the results obtained by Doughman et al. (19) showing that PIP5Kα kinase activity is essential for platelet-derived growth factor– and Rac1-mediated actin remodeling in fibroblasts. However, in T lymphocytes,

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

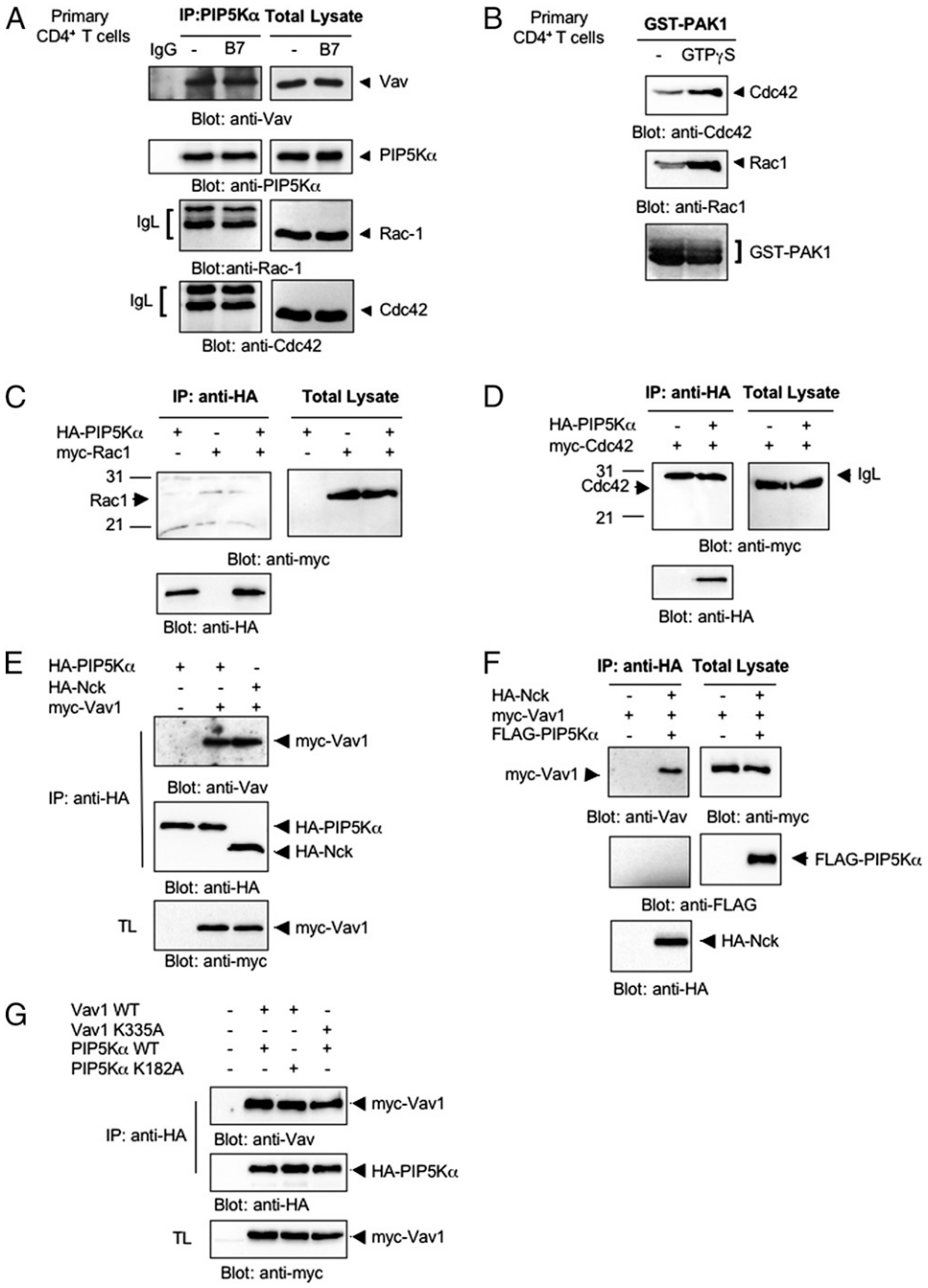

FIGURE 7. PIP5Kα associates with Vav1 in a complex containing Nck. (**A**) Primary CD4$^+$ T cells were stimulated for 5 min with adherent B7-negative cells (−) or Dap/B7 (B7) cells. Anti-PIP5Kα, anti-Vav, anti-PIP5Kα, anti-Rac1, and anti-Cdc42 Western blottings were performed on anti-PIP5Kα or isotype-matched control IgG or total lysates. The results are representative of three independent experiments. (**B**) Total lysates of primary CD4$^+$ T cells were incubated with or without (−) guanosine 5′-*O*-[γ-thio] triphosphate (GTPγS), and the association of Cdc42 or Rac1 with PAK1 was analyzed in pulldown assays using GST-PAK1 beads. CD28 WT cells were transfected with HA-PIP5Kα alone or in combination with myc-Rac1 (**C**) or myc-Cdc42 (**D**) vectors. Anti-myc and anti-HA Western blottings were performed on anti-HA immunoprecipitation (IP) and/or total lysates. The m.w. markers position are indicated on the *left*. (**E**) CD28 WT cells were transfected with the indicated expression vectors, and anti-myc, anti-Vav, or anti-HA Western blottings were performed on anti-HA IP and/or total lysates (TL). (**F**) CD28 WT cells were transfected with the indicated expression vectors, and anti-Vav, anti-myc, anti-FLAG, or anti-HA Western blottings were performed on anti-HA IP and/or total lysates. The results are representative of three independent experiments. (**G**) CD28 WT cells were transfected with the indicated expression vectors, and anti-Vav, anti-myc, or anti-HA Western blottings were performed on anti-HA IP and/or TL. The results are representative of three independent experiments.

PIP5Kα does not interact with Rac1 or Cdc42. Instead, we found that PIP5Kα associates with Vav1 (Fig. 7). We also evidence that PIP5Kα activity is pivotal for Vav1-regulated signaling pathways downstream of CD28 (Fig. 3B).

Vav1 is a critical signal transducer of both TCR and CD28 molecules. It controls the downstream signaling pathways triggered by both TCR and CD28 as well as cytoskeleton reorganization events necessary for cell polarization and IS formation (45, 68). Vav1 is also a critical mediator of CD28 autonomous signaling leading to NFAT, NF-κB activation, and IL-2/IL-4 transcription in a TCR-dependent manner (35, 37, 69). These critical signaling functions of Vav1 have been reported to depend on its GEF activity. Consistently, we found that the overexpression of Vav1 (K335A) mutant, defective in its GEF activity (50), impairs CD28-induced PIP5Kα recruitment to the T–APC contact site (Fig. 4). Furthermore, PIP5Kα kinase activity appears to be fundamental for Vav1 effector functions as the overexpression of the PIP5Kα (K182A) kinase-dead mutant significantly reduced CD28-dependent Vav1 recruitment and signaling functions (Figs. 3, 4). These data evidence a reciprocal interdependence between Vav1 and PIP5Kα in promoting the actin polymerization events required for the efficient recruitment and activation of essential signaling complexes. Moreover, we also confirmed the cooperative interaction of Vav1 with Nckβ (63) (Fig. 7). Nckβ adapter plays an important role in mediating actin cytoskeleton reorganization (70) and a synergistic relationship between Nckβ and PIP2 in promoting N-WASp–dependent actin rearrangements in living cells has been also evidenced (64). Our data strongly support a critical role of Vav1 in connecting PIP2 and PIP5Kα to localized Nck/WASp-mediated actin polymerization. The analyses of the molecular basis of PIP5Kα/Vav1 association as well as the identification of Vav1 domains involved in binding PIP5Kα are in progress.

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

The key feature of CD28 signal capability derives from the sequence of its small cytoplasmic domain (41 aa) (47). We evidenced that the C-terminal motif of CD28 is critically involved in the dynamics of actin polymerization (Supplemental Fig. 4A) and in the recruitment of both PIP5Kα (32) and Vav1 (Fig. 5), but not in CD28 downregulation (Supplemental Fig. 4B). On the basis of the ability of Vav1 to interact with Grb2 (59, 60), Grb2 binding to CD28 has been also suggested as the mechanism by which CD28 recruits Vav1 (58). Interestingly, in addition to bind the YMNM motif through its SH2 domain, Grb2 interacts with CD28 by binding the C-terminal motif YQPYAPP (54, 61, 71). However, when we verified the existence of Vav1/Grb2 complexes or the recruitment of Grb2 to CD28 in human primary $CD4^+$ T cells, we did not find any association neither between CD28 and Grb2 (Fig. 6B) nor between Vav1 and Grb2 (Fig. 6C). The reason for these discrepancies may be due to the fact that all data on CD28/Grb2 as well as on Vav1/Grb2 association were obtained in mouse cells, in in vitro binding assays, or following overexpression of exogenous proteins in different cell lines. Conversely, we found that, in human primary $CD4^+$ T cells and Jurkat cells stimulated by B7, in the absence of TCR stimulation, CD28 associates with Vav1 through its C-terminal YQPYAPP (Fig. 6D) but not through the YMNM that selectively binds the p85 subunit of PI3K (Supplemental Fig. 2B). Moreover, our data on the loss of CD28/Vav1 interaction by mutating the tyrosine residues within the C-terminal proline-rich motif of CD28 (Fig. 6D) suggest a role for the SH2 domain of Vav1. Considering the different consensus sequences (e.g., **Y**ES**P**, **Y**EE**P**, **Y**ME**P**, **Y**AD**P**, **Y**LN**P**) identified as optimal binding sites for the SH2 of Vav1 (48, 72–75), the phosphorylated $\mathbf{Y}^{209}$A**P**$\mathbf{P}^{212}$ within the C-terminal proline-rich motif of CD28 may likely represent an optimal binding motif. Alternatively, a CD28- and Vav1-binding partner may function as a linker, thus favoring CD28/Vav1 interaction. Experiments are in progress to verify this hypothesis.

In conclusion, our data provide evidence that Vav1 is the linker molecule that couples CD28 to PIP5Kα activation and strongly fit with a potential model in which CD28 regulates PIP2 synthesis and turnover in T lymphocytes: 1) by binding Vav1, the C-terminal proline-rich motif of CD28 recruits PIP5Kα, which in turn synthesizes PIP2, a crucial source for both actin polymerization and second messenger generation; and 2) the YMNM motif recruits and activates class IA PI3K that phosphorylates PIP2 to generate PIP3 lipids, which are the docking sites for Vav1 and important mediators of both CD28 autonomous and TCR costimulatory signals (Supplemental Fig 3).

Acknowledgments

We thank Giovanni L. Buglia (Department of Biology and Biotechnology "Charles Darwin," Sapienza University) for assistance in confocal analyses. We also thank D. Cantrell (University of Dundee, Dundee, U.K.), A. Hall (Memorial Sloan-Kettering Cancer Center, New York, NY), W. Li (University of Southern California, Los Angeles, Los Angeles, CA), J.F. Peyron (Faculté de Médicine Pasteur, Nice, France), G.A. Koretzky (Weill Cornell Medical College, New York, NY), and C. Baldari (University of Siena, Siena, Italy) for reagents.

Disclosures

The authors have no financial conflicts of interest.

References

1. Acuto, O., and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat. Rev. Immunol.* 3: 939–951.
2. Rudd, C. E., and M. Raab. 2003. Independent CD28 signaling via VAV and SLP-76: a model for in trans costimulation. *Immunol. Rev.* 192: 32–41.
3. Camperio, C., M. Muscolini, E. Volpe, D. Di Mitri, R. Mechelli, M. C. Buscarinu, S. Ruggieri, E. Piccolella, M. Salvetti, C. Gasperini, et al. 2014. CD28 ligation in the absence of TCR stimulation up-regulates IL-17A and pro-inflammatory cytokines in relapsing-remitting multiple sclerosis T lymphocytes. *Immunol. Lett.* 158: 134–142.
4. Cianfrocca, R., M. Muscolini, V. Marzano, A. Annibaldi, B. Marinari, M. Levrero, A. Costanzo, and L. Tuosto. 2008. RelA/NF-kappaB recruitment on the bax gene promoter antagonizes p73-dependent apoptosis in costimulated T cells. *Cell Death Differ.* 15: 354–363.
5. Kovacs, B., R. V. Parry, Z. Ma, E. Fan, D. K. Shivers, B. A. Freiberg, A. K. Thomas, R. Rutherford, C. A. Rumbley, J. L. Riley, and T. H. Finkel. 2005. Ligation of CD28 by its natural ligand CD86 in the absence of TCR stimulation induces lipid raft polarization in human CD4 T cells. *J. Immunol.* 175: 7848–7854.
6. Marinari, B., A. Costanzo, V. Marzano, E. Piccolella, and L. Tuosto. 2004. CD28 delivers a unique signal leading to the selective recruitment of RelA and p52 NF-kappaB subunits on IL-8 and Bcl-xL gene promoters. *Proc. Natl. Acad. Sci. USA* 101: 6098–6103.
7. Acuto, O., and D. Cantrell. 2000. T cell activation and the cytoskeleton. *Annu. Rev. Immunol.* 18: 165–184.
8. Ritter, A. T., K. L. Angus, and G. M. Griffiths. 2013. The role of the cytoskeleton at the immunological synapse. *Immunol. Rev.* 256: 107–117.
9. Saarikangas, J., H. Zhao, and P. Lappalainen. 2010. Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. *Physiol. Rev.* 90: 259–289.
10. Rapley, J., V. L. Tybulewicz, and K. Rittinger. 2008. Crucial structural role for the PH and C1 domains of the Vav1 exchange factor. *EMBO Rep.* 9: 655–661.
11. Zhang, T. T., H. Li, S. M. Cheung, J. L. Costantini, S. Hou, M. Al-Alwan, and A. J. Marshall. 2009. Phosphoinositide 3-kinase-regulated adapters in lymphocyte activation. *Immunol. Rev.* 232: 255–272.
12. Mao, Y. S., and H. L. Yin. 2007. Regulation of the actin cytoskeleton by phosphatidylinositol 4-phosphate 5 kinases. *Pflugers Arch.* 455: 5–18.
13. van den Bout, I., and N. Divecha. 2009. PIP5K-driven PtdIns(4,5)P2 synthesis: regulation and cellular functions. *J. Cell Sci.* 122: 3837–3850.
14. Ishihara, H., Y. Shibasaki, N. Kizuki, H. Katagiri, Y. Yazaki, T. Asano, and Y. Oka. 1996. Cloning of cDNAs encoding two isoforms of 68-kDa type I phosphatidylinositol-4-phosphate 5-kinase. *J. Biol. Chem.* 271: 23611–23614.
15. Ishihara, H., Y. Shibasaki, N. Kizuki, T. Wada, Y. Yazaki, T. Asano, and Y. Oka. 1998. Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. *J. Biol. Chem.* 273: 8741–8748.
16. Loijens, J. C., and R. A. Anderson. 1996. Type I phosphatidylinositol-4-phosphate 5-kinases are distinct members of this novel lipid kinase family. *J. Biol. Chem.* 271: 32937–32943.
17. Sun, Y., R. D. Dandekar, Y. S. Mao, H. L. Yin, and C. Wulfing. 2011. Phosphatidylinositol (4,5) bisphosphate controls T cell activation by regulating T cell rigidity and organization. *PLoS ONE* 6: e27227.
18. Chatah, N. E., and C. S. Abrams. 2001. G-protein-coupled receptor activation induces the membrane translocation and activation of phosphatidylinositol-4-phosphate 5-kinase I alpha by a Rac- and Rho-dependent pathway. *J. Biol. Chem.* 276: 34059–34065.
19. Doughman, R. L., A. J. Firestone, M. L. Wojtasiak, M. W. Bunce, and R. A. Anderson. 2003. Membrane ruffling requires coordination between type Ialpha phosphatidylinositol phosphate kinase and Rac signaling. *J. Biol. Chem.* 278: 23036–23045.
20. Barbieri, M. A., C. M. Heath, E. M. Peters, A. Wells, J. N. Davis, and P. D. Stahl. 2001. Phosphatidylinositol-4-phosphate 5-kinase-1beta is essential for epidermal growth factor receptor-mediated endocytosis. *J. Biol. Chem.* 276: 47212–47216.
21. Coppolino, M. G., M. Krause, P. Hagendorff, D. A. Monner, W. Trimble, S. Grinstein, J. Wehland, and A. S. Sechi. 2001. Evidence for a molecular complex consisting of Fyb/SLAP, SLP-76, Nck, VASP and WASP that links the actin cytoskeleton to Fc{gamma} receptor signalling during phagocytosis. *J. Cell Sci.* 114: 4307–4318.
22. Micucci, F., C. Capuano, E. Marchetti, M. Piccoli, L. Frati, A. Santoni, and R. Galandrini. 2008. PI5KI-dependent signals are critical regulators of the cytolytic secretory pathway. *Blood* 111: 4165–4172.
23. Kaga, S., S. Ragg, K. A. Rogers, and A. Ochi. 1998. Stimulation of CD28 with B7-2 promotes focal adhesion-like contacts where Rho family small G proteins accumulate in T cells. *J. Immunol.* 160: 24–27.
24. Kaga, S., S. Ragg, K. A. Rogers, and A. Ochi. 1998. Activation of p21-CDC42/Rac-activated kinases by CD28 signaling: p21-activated kinase (PAK) and MEK kinase 1 (MEKK1) may mediate the interplay between CD3 and CD28 signals. *J. Immunol.* 160: 4182–4189.
25. Michel, F., G. Attal-Bonnefoy, G. Mangino, S. Mise-Omata, and O. Acuto. 2001. CD28 as a molecular amplifier extending TCR ligation and signaling capabilities. *Immunity* 15: 935–945.
26. Tavano, R., R. L. Contento, S. J. Baranda, M. Soligo, L. Tuosto, S. Manes, and A. Viola. 2006. CD28 interaction with filamin-A controls lipid raft accumulation at the T-cell immunological synapse. *Nat. Cell Biol.* 8: 1270–1276.
27. Villalba, M., N. Coudronniere, M. Deckert, E. Teixeiro, P. Mas, and A. Altman. 2000. A novel functional interaction between Vav and PKCtheta is required for TCR-induced T cell activation. *Immunity* 12: 151–160.
28. Wulfing, C., and M. M. Davis. 1998. A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science* 282: 2266–2269.
29. Salazar-Fontana, L. I., V. Barr, L. E. Samelson, and B. E. Bierer. 2003. CD28 engagement promotes actin polymerization through the activation of the small Rho GTPase Cdc42 in human T cells. *J. Immunol.* 171: 2225–2232.
30. Badour, K., M. K. McGavin, J. Zhang, S. Freeman, C. Vieira, D. Filipp, M. Julius, G. B. Mills, and K. A. Siminovitch. 2007. Interaction of the Wiskott-Aldrich syndrome protein with sorting nexin 9 is required for CD28 endocytosis and cosignaling in T cells. *Proc. Natl. Acad. Sci. USA* 104: 1593–1598.

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015

31. Seminario, M. C., and R. L. Wange. 2003. Lipid phosphatases in the regulation of T cell activation: living up to their PTEN-tial. *Immunol. Rev.* 192: 80–97.
32. Muscolini, M., C. Camperio, C. Capuano, S. Caristi, E. Piccolella, R. Galandrini, and L. Tuosto. 2013. Phosphatidylinositol 4-phosphate 5-kinase alpha activation critically contributes to CD28-dependent signaling responses. *J. Immunol.* 190: 5279–5286.
33. Hewitt, C. R., J. R. Lamb, J. Hayball, M. Hill, M. J. Owen, and R. E. O'Hehir. 1992. Major histocompatibility complex independent clonal T cell anergy by direct interaction of *Staphylococcus aureus* enterotoxin B with the T cell antigen receptor. *J. Exp. Med.* 175: 1493–1499.
34. Muscolini, M., A. Sajeva, S. Caristi, and L. Tuosto. 2011. A novel association between filamin A and NF-kappaB inducing kinase couples CD28 to inhibitor of NF-kappaB kinase alpha and NF-kappaB activation. *Immunol. Lett.* 36: 203–212.
35. Piccolella, E., F. Spadaro, C. Ramoni, B. Marinari, A. Costanzo, M. Levrero, L. Thomson, R. T. Abraham, and L. Tuosto. 2003. Vav-1 and the IKK alpha subunit of I kappa B kinase functionally associate to induce NF-kappa B activation in response to CD28 engagement. *J. Immunol.* 170: 2895–2903.
36. Tuosto, L., and O. Acuto. 1998. CD28 affects the earliest signaling events generated by TCR engagement. *Eur. J. Immunol.* 28: 2132–2142.
37. Michel, F., G. Mangino, G. Attal-Bonnefoy, L. Tuosto, A. Alcover, A. Roumier, D. Olive, and O. Acuto. 2000. CD28 utilizes Vav-1 to enhance TCR-proximal signaling and NF-AT activation. *J. Immunol.* 165: 3820–3829.
38. Marinari, B., A. Costanzo, A. Viola, F. Michel, G. Mangino, O. Acuto, M. Levrero, E. Piccolella, and L. Tuosto. 2002. Vav cooperates with CD28 to induce NF-kappaB activation via a pathway involving Rac-1 and mitogen-activated kinase kinase 1. *Eur. J. Immunol.* 32: 447–456.
39. Chen, M., H. She, E. M. Davis, C. M. Spicer, L. Kim, R. Ren, M. M. Le Beau, and W. Li. 1998. Identification of Nck family genes, chromosomal localization, expression, and signaling specificity. *J. Biol. Chem.* 273: 25171–25178.
40. Galandrini, R., F. Micucci, I. Tassi, M. G. Cifone, B. Cinque, M. Piccoli, L. Frati, and A. Santoni. 2005. Arf6: a new player in FcgammaRIIIA lymphocyte-mediated cytotoxicity. *Blood* 106: 577–583.
41. Valitutti, S., M. Dessing, K. Aktories, H. Gallati, and A. Lanzavecchia. 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J. Exp. Med.* 181: 577–584.
42. Tolias, K. F., J. H. Hartwig, H. Ishihara, Y. Shibasaki, L. C. Cantley, and C. L. Carpenter. 2000. Type Ialpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr. Biol.* 10: 153–156.
43. Tan, Y. X., B. N. Manz, T. S. Freedman, C. Zhang, K. M. Shokat, and A. Weiss. 2014. Inhibition of the kinase Csk in thymocytes reveals a requirement for actin remodeling in the initiation of full TCR signaling. *Nat. Immunol.* 15: 186–194.
44. Céfaï, D., H. Schneider, O. Matangkasombut, H. Kang, J. Brody, and C. E. Rudd. 1998. CD28 receptor endocytosis is targeted by mutations that disrupt phosphatidylinositol 3-kinase binding and costimulation. *J. Immunol.* 160: 2223–2230.
45. Tybulewicz, V. L. J. 2005. Vav-family proteins in T-cell signalling. *Curr. Opin. Immunol.* 17: 267–274.
46. Michel, F., and O. Acuto. 2002. CD28 costimulation: a source of Vav-1 for TCR signaling with the help of SLP-76? *Sci. STKE* 144: pe35.
47. Tuosto, L. 2011. NF-kappaB family of transcription factors: biochemical players of CD28 co-stimulation. *Immunol. Lett.* 135: 1–9.
48. Tuosto, L., F. Michel, and O. Acuto. 1996. p95vav associates with tyrosine-phosphorylated SLP-76 in antigen-stimulated T cells. *J. Exp. Med.* 184: 1161–1166.
49. Wu, J., D. G. Motto, G. A. Koretzky, and A. Weiss. 1996. Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. *Immunity* 4: 593–602.
50. Saveliev, A., L. Vanes, O. Ksionda, J. Rapley, S. J. Smerdon, K. Rittinger, and V. L. Tybulewicz. 2009. Function of the nucleotide exchange activity of vav1 in T cell development and activation. *Sci. Signal.* 2: ra83.
51. Cai, Y. C., D. Cefai, H. Schneider, M. Raab, N. Nabavi, and C. E. Rudd. 1995. Selective CD28pYMNM mutations implicate phosphatidylinositol 3-kinase in CD86-CD28-mediated costimulation. *Immunity* 3: 417–426.
52. Prasad, K. V., Y. C. Cai, M. Raab, B. Duckworth, L. Cantley, S. E. Shoelson, and C. E. Rudd. 1994. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc. Natl. Acad. Sci. USA* 91: 2834–2838.
53. Truitt, K. E., C. M. Hicks, and J. B. Imboden. 1994. Stimulation of CD28 triggers an association between CD28 and phosphatidylinositol 3-kinase in Jurkat T cells. *J. Exp. Med.* 179: 1071–1076.
54. Okkenhaug, K., and R. Rottapel. 1998. Grb2 forms an inducible protein complex with CD28 through a Src homology 3 domain-proline interaction. *J. Biol. Chem.* 273: 21194–21202.
55. Schneider, H., Y. C. Cai, K. V. Prasad, S. E. Shoelson, and C. E. Rudd. 1995. T cell antigen CD28 binds to the GRB-2/SOS complex, regulators of p21ras. *Eur. J. Immunol.* 25: 1044–1050.
56. August, A., S. Gibson, Y. Kawakami, T. Kawakami, G. B. Mills, and B. Dupont. 1994. CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. *Proc. Natl. Acad. Sci. USA* 91: 9347–9351.
57. Holdford, A. D., J. M. Green, S. D. Levin, M. F. Denny, D. B. Straus, P. S. Link, P. S. Changelian, P. M. Allen, and A. S. Shaw. 1999. Proline residues in CD28 and the Src homology (SH)3 domain of Lck are required for T cell costimulation. *J. Exp. Med.* 190: 375–384.
58. Schneider, H., and C. E. Rudd. 2008. CD28 and Grb-2, relative to Gads or Grap, preferentially co-operate with Vav1 in the activation of NFAT/AP-1 transcription. *Biochem. Biophys. Res. Commun.* 369: 616–621.
59. Nishida, M., K. Nagata, Y. Hachimori, M. Horiuchi, K. Ogura, V. Mandiyan, J. Schlessinger, and F. Inagaki. 2001. Novel recognition mode between Vav and Grb2 SH3 domains. *EMBO J.* 20: 2995–3007.
60. Ramos-Morales, F., B. J. Druker, and S. Fischer. 1994. Vav binds to several SH2/SH3 containing proteins in activated lymphocytes. *Oncogene* 9: 1917–1923.
61. Kim, H. H., M. Tharayil, and C. E. Rudd. 1998. Growth factor receptor-bound protein 2 SH2/SH3 domain binding to CD28 and its role in co-signaling. *J. Biol. Chem.* 273: 296–301.
62. Coppolino, M. G., R. Dierckman, J. Loijens, R. F. Collins, M. Pouladi, J. Jongstra-Bilen, A. D. Schreiber, W. S. Trimble, R. Anderson, and S. Grinstein. 2002. Inhibition of phosphatidylinositol-4-phosphate 5-kinase Ialpha impairs localized actin remodeling and suppresses phagocytosis. *J. Biol. Chem.* 277: 43849–43857.
63. Barda-Saad, M., N. Shirasu, M. H. Pauker, N. Hassan, O. Perl, A. Balbo, H. Yamaguchi, J. C. Houtman, E. Appella, P. Schuck, and L. E. Samelson. 2010. Cooperative interactions at the SLP-76 complex are critical for actin polymerization. *EMBO J.* 29: 2315–2328.
64. Rivera, G. M., D. Vasilescu, V. Papayannopoulos, W. A. Lim, and B. J. Mayer. 2009. A reciprocal interdependence between Nck and PI(4,5)P(2) promotes localized N-WASp-mediated actin polymerization in living cells. *Mol. Cell* 36: 525–535.
65. Boomer, J. S., and J. M. Green. 2010. An enigmatic tail of CD28 signaling. *Cold Spring Harb. Perspect. Biol.* 2: a002436.
66. Capuano, C., R. Paolini, R. Molfetta, L. Frati, A. Santoni, and R. Galandrini. 2012. PIP2-dependent regulation of Munc13-4 endocytic recycling: impact on the cytolytic secretory pathway. *Blood* 119: 2252–2262.
67. Singleton, K. L., K. T. Roybal, Y. Sun, G. Fu, N. R. Gascoigne, N. S. van Oers, and C. Wulfing. 2009. Spatiotemporal patterning during T cell activation is highly diverse. *Sci. Signal.* 2: ra15.
68. Ksionda, O., A. Saveliev, R. Kochl, J. Rapley, M. Faroudi, J. E. Smith-Garvin, C. Wulfing, K. Rittinger, T. Carter, and V. L. Tybulewicz. 2012. Mechanism and function of Vav1 localisation in TCR signalling. *J. Cell Sci.* 125: 5302–5314.
69. Raab, M., S. Pfister, and C. E. Rudd. 2001. CD28 signaling via VAV/SLP-76 adaptors: regulation of cytokine transcription independent of TCR ligation. *Immunity* 15: 921–933.
70. Buday, L., L. Wunderlich, and P. Tamás. 2002. The Nck family of adapter proteins: regulators of actin cytoskeleton. *Cell. Signal.* 14: 723–731.
71. Higo, K., M. Oda, H. Morii, J. Takahashi, Y. Harada, S. Ogawa, and R. Abe. 2014. Quantitative analysis by surface plasmon resonance of CD28 interaction with cytoplasmic adaptor molecules Grb2, Gads and p85 PI3K. *Immunol. Invest.* 43: 278–291.
72. Chen, C. H., D. Piraner, N. M. Gorenstein, R. L. Geahlen, and C. Beth Post. 2013. Differential recognition of syk-binding sites by each of the two phosphotyrosine-binding pockets of the Vav SH2 domain. *Biopolymers* 99: 897–907.
73. Huang, H., L. Li, C. Wu, D. Schibli, K. Colwill, S. Ma, C. Li, P. Roy, K. Ho, Z. Songyang, et al. 2008. Defining the specificity space of the human SRC homology 2 domain. *Mol. Cell. Proteomics* 7: 768–784.
74. O'Rourke, L. M., R. Tooze, M. Turner, D. M. Sandoval, R. H. Carter, V. L. Tybulewicz, and D. T. Fearon. 1998. CD19 as a membrane-anchored adaptor protein of B lymphocytes: costimulation of lipid and protein kinases by recruitment of Vav. *Immunity* 8: 635–645.
75. Songyang, Z., S. E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X. R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, T. Yi, et al. 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.* 14: 2777–2785.

Downloaded from http://www.jimmunol.org/ at Univ Studi La Sapienza on January 19, 2015