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CD28 and Lipid Rafts Coordinate Recruitment of Lck to the Immunological Synapse of Human T Lymphocytes[1]

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In T lymphocytes, the Src family kinase Lck associates lipid rafts and accumulates at the immunological synapse (IS) during T cell stimulation by APCs. Using CD4- or CD28-deficient murine T cells, it was suggested that recruitment of Lck to the IS depends on CD4, whereas CD28 sustains Lck activation. However, in human resting T cells, CD28 is responsible for promoting recruitment of lipid rafts to the IS by an unknown mechanism. Thus, we performed a series of experiments to determine 1) whether Lck is recruited to the IS through lipid rafts; and 2) whether Lck recruitment to the IS of human resting T cells depends on CD4 or on CD28 engagement. We found that CD28, but not CD4, stimulation induced recruitment of Lck into detergent-resistant domains as well as its accumulation at the IS. We also found that Lck recruitment to the IS depends on the CD28 COOH-terminal PxxPP motif. Thus, the CD28-3A mutant, generated by substituting the prolines in positions 208, 211, and 212 with alanines, failed to induce Lck and lipid raft accumulation at the synapse. These results indicate that CD28 signaling orchestrates both Lck and lipid raft recruitment to the IS to amplify T cell activation. *The Journal of Immunology,* **2004, 173: 5392–5397.**

In naive T lymphocytes, the outcome of TCR stimulation is regulated by the simultaneous binding of CD28 to its natural ligand, B7, expressed on APCs. CD28 costimulation enhances the production of cytokines, such as IL-2, prevents anergy and programmed cell death, and finally determines the fate of an antigenic stimulation (1). Thus, mice lacking CD28 can still mount T cell responses, but require repeated stimulations with high doses of Ag (2). In vitro studies demonstrated that in the absence of CD28 engagement, T cells require a very high TCR occupancy and a prolonged stimulation, whereas when costimulated via CD28, they respond more rapidly to lower levels of TCR occupancy (3, 4). Several reports demonstrated that CD28 can specifically enhance distinct TCR signaling pathways, indicating that CD28 acts as an amplifier of early TCR signaling (5).

In addition to activating specific signals integrating those delivered through TCR, CD28 may act as an independent signaling unit able to furnish in trans costimulation (1). Indeed, independently of TCR, CD28 stimulation induces cytoskeletal rearrangements (6), up-regulation of IL-2 and IL-4 transcription (7), as well as IκB kinase and NF-κB activation (8). CD28 stimulation by Abs or B7 results in tyrosine phosphorylation of specific cellular substrates, including Vav-1, PI3K, and the intracellular domain of CD28 itself (5, 9). Due to the absence of an intrinsic kinase activity, the CD28-induced increase in tyrosine phosphorylation is probably due to the recruitment and/or activation of tyrosine kinases. Both the thymus-expressed chemokine family tyrosine kinase IL-2-inducible T cell kinase (ITK)[4] (10) and the Src family kinase Lck (11) are activated after CD28 stimulation. ITK association with CD28 after CD28 stimulation is involved in generating both positive and negative signaling (11–13). Lck mediates the tyrosine phosphorylation of CD28 itself (14) as well as the activation of ITK (15).

It was suggested that plasma membranes contain specialized lipid microdomains, called rafts. The raft hypothesis predicts that attractive forces between sphingolipids and cholesterol mediate the formation of small and transient lipid clusters in an unsaturated glycerolphospholipid environment (16, 17). The biochemical techniques used for rafts analysis (i.e., their isolation after cell membrane solubilization by mild detergents) have shown that many lipid-modified signaling proteins, such as tyrosine kinases of the Src family, GPI-linked proteins, and adaptor proteins, are concentrated in raft domains. This selective confinement of signaling molecules in membrane subdomains has suggested that lipid rafts could function as platforms for the formation of multicomponent transduction complexes.

In T cells, rafts are enriched in many signaling molecules and participate in TCR triggering and T cell activation (18). Thus, the TCR, which under resting conditions is excluded or weakly associated to rafts, after engagement translocates into raft domains, where supramolecular complexes containing phosphorylated signaling molecules are accumulated (19). Moreover, activation of resting peripheral blood (PB) T cells is accompanied by lipid raft polarization to the receptor engagement site (20), an event regulated by CD28 costimulation and probably dependent on actin cytoskeleton reorganization (21).

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[4] Abbreviations used in this paper: ITK, IL-2-inducible T cell kinase; DRM, detergent-resistant membrane microdomain; IS, immunological synapse; MFI, mean fluorescence intensity; MyrPalm-mCFP, cyan fluorescent protein carrying consensus sequences for myristoylation plus palmitoylation; PB, peripheral blood; SAG, bacterial superantigen; SEE, staphylococcal enterotoxin E; WT, wild type.

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Lck is a typical raft-preferring molecule, and in many studies it has been used as a raft marker (22). However, it was suggested that CD4, not CD28, engagement determines the recruitment of Lck to the immunological synapse (IS) (23). Moreover, conflicting results on the roles of CD28 and lipid rafts in the organization of the IS have been reported in the last few years (20, 24, 25). Therefore, we analyzed the roles of CD4 and CD28 in Lck recruitment to the IS of human resting PB T cells. In this study we show that CD28, but not CD4, engagement induces Lck recruitment into lipid rafts and accumulation of the kinase at IS, by a mechanism dependent on Vav-1 and requiring the CD28 binding motif for Lck.

Materials and Methods

Cell lines, Abs, and reagents

The $CD4^+$ Jurkat T cell line and the Vav-1 deficient cell line (J.Vav1; provided by R. T. Abraham, The Burnham Institute, La Jolla, CA) (26) were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin (Invitrogen Life Technologies, Gaithersburg, MD). The CD28-negative Jurkat T cell line CH7C17 (provided by O. Acuto, Department of Immunology, Institut Pasteur, Paris, France) was maintained as described above with the addition of 400 μg/ml hygromycin B and 4 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO). The J.Vav1 derivative cell line, J.Vav1 wild type (J.Vav1WT), stably re-expressing Vav-1 was maintained as described above with the addition of 2 mg/ml G418. The L cell transfectants expressing human HLA-DRB1*0101 (5-3.1) or cotransfected with HLA-DRB1*0101 and human B7.1 (5-3.1/B7) were previously described (8). Human PB $CD4^+$ T cells were purified by negative selection using an indirect MACS kit (Miltenyi Biotec, Auburn, CA). Mouse anti-Lck (3A5) and goat anti-CD3ϵ (M20) antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-CD4 (S3.5) was obtained from Caltag Laboratories (Burlingame, CA). Anti-human CD28 Ab (CD28.2) and anti-human CD71 (M-A712) were purchased from BD Pharmingen (Milan, Italy). Staphylococcal enterotoxins A, B, and E (SEE) were purchased from Toxin Technology (Sarasota, FL).

Plasmid and transfections

PEF-Bos was provided by D. Cantrell (London, U.K.). The cDNA encoding human CD28WT (27) were subcloned in pEF-Bos vector. CD28-3A mutant was derived from CD28WT by substituting Pro^{208}, Pro^{211}, and Pro^{212} in the COOH-terminal PxxPP motif of human CD28 to alanines by PCR. The primers used were 5′-TACCAGGCCTATGCCGCAGCAC GAGAC-3′ and 5′-GTCTCGTGCTGCGGCATAGGCGTGGTA-3′. The entire sequence of the mutant was verified by DNA sequencing. Stable transfectants were obtained by electroporating (at 260 V, 960 μF) 10^7 CH7C17 cells in 0.5 ml of RPMI 1640 supplemented with 20% FCS with 30 μg of pEF-Bos-neo-encoding CD28WT or CD28-3A. After 48 h, cells were placed in 96-well culture plates in selective RPMI 1640 medium containing 2 mg/ml G418 (Sigma-Aldrich). Transfectants were subjected to cell sorting by FACScan (BD Biosciences, San Jose, CA) to obtain stable cell lines expressing similar levels of CD28.

Cyan fluorescent protein carrying consensus sequences for myristoylation plus palmitoylation (MyrPalm-mCFP) in pcDNA3 plasmid, coding for lipid-modified fluorescent proteins, was a gift from R. Tsien (San Diego, CA). cDNA constructs were transfected by electroporation using 50 μg of DNA (20 μg of MyrPalm-mCFP plus 30 μg of pcDNA3) and 10^7 Jurkat cells suspended in 400 μl of RPMI 1640 supplemented with 20% FCS. Electroporation was performed in 0.45-cm electroporation cuvettes (Gene Pulser; Bio-Rad, Hercules, CA) at 960 μF and 250 V. After 24 h, transfected cells were diluted and selected in 2 mg/ml geneticin-containing medium (G418; Invitrogen Life Technologies). Human PB $CD4^+$ T cells were transiently transfected using an electroporation system (Amaxa Biosystems, Koeln, Germany) according to the manufacturer's guidelines and were used for experiments 24 h later.

Preparation of detergent-resistant membrane microdomains (DRM)

CD28 or CD4 stimulation was performed by incubating 5×10^6 cells/ml in 1% FCS-supplemented medium in the presence of 10 μg/ml anti-CD28 or anti-CD4 Abs for 30 min at 4°C. Cells were washed, resuspended and cross-linked with 5 μg/ml anti-mouse Abs for 15 min at 37°C. Stimulated or unstimulated cells (25×10^6) were washed twice with ice-cold medium and homogenized in 1.5 ml of ice-cold 0.15 M NaCl and 25 mM MES, pH 6.5, containing 0.2% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, and 50 mM sodium fluoride. The homogenates were incubated for 1 h on ice under gentle shaking and then either centrifuged for 5 min at $400 \times g$ to remove nuclei and debris or directly subjected to the density gradient centrifugation (we found no differences using the two different methods). The supernatants (~1.5 ml) were then adjusted to 45% sucrose by the addition of 1.5 ml of 90% sucrose/0.15 M NaCl and 25 mM MES, pH 6.5, placed in the bottom of ultracentrifuge tubes. A 5–35% discontinuous sucrose gradient was formed above (3 ml of 5% sucrose/6 ml of 35% sucrose). The gradients were centrifuged at $187{,}000 \times g$ in a SW40 Ti rotor (Beckman Coulter, Fullerton, CA) for 20 h at 4°C. Twelve fractions (1 ml each) were collected from the top of the gradients and loaded onto 12% polyacrylamide gels and blotted onto nitrocellulose. The immunoblots were probed with anti-Lck (3A5; Santa Cruz Biotechnology) or anti-CD3ϵ (M20; Santa Cruz Biotechnology) Abs. Secondary Abs HRP-conjugated were obtained from Bio-Rad. The immunoblots were analyzed by ECL (Amersham Biosciences, Arlington Heights, IL).

Confocal microscopy

5-3.1 and 5-3.1/B7 cells were suspended at 10^7 cells/ml and incubated (or not) with 1 μg/ml of the bacterial superantigen SEE or with a mixture of superantigens (staphylococcal enterotoxin A, staphylococcal enterotoxin B, and SEE; 1 μg/ml each) for 2 h at 37°C, mixing every 20 min. Pulsed cells were washed and incubated for 15 min at 37°C with equal amount of MyrPalm-mCFP-expressing T cells. Cells were then adhered to microscope slides coated with 50 μg/ml poly-L-lysine, fixed with 2% paraformaldehyde, and either permeabilized with 0.1% Triton/PBS and stained for Lck or directly mounted in 2.5% 1,4-diazobicyclo[2.2.2]octane (Fluka, Buchs, Switzerland), 90% glycerol, and 10% PBS.

Confocal microscopy was performed with a confocal microscope (Bio-Rad) with ×60 and ×100 objective lenses (Nikon, Melville, NY), using laser excitation at 488 nm. Images were analyzed with the Photoshop 7.0 program (Adobe Systems, San Jose, CA). Surface plot analysis was performed with the program Image J.

Quantitation of fluorescence in microscopic images

To quantitate the recruitment of Lck and MyrPalm-mCFP to the IS, boxes of equal area were drawn around the IS, the regions of the T cell not in contact with APC, and a background area outside of the cell. The relative recruitment index was calculated as indicated: [mean fluorescence intensity (MFI) at synapse − background]/[MFI at regions not in contact with APC − background]. Quantitative analysis of MFI was performed with the program Image J. At least 30 cells or conjugates were examined quantitatively for each experiment. Statistical significance was calculated by Student's *t* test. As a control for membrane ruffling, the relative recruitment index of CD71 and the transmembrane protein LGFPGT46 was calculated and in all the experimental conditions was ≤ 1.

Densitometry

Densitometric analyses were performed on an Image Master VDS-CL densitometer using volume analysis of Image Master Total Lab software (Amersham Biosciences). All densitometric values obtained were calculated from nonsaturated signals.

Results

CD28 stimulation induces recruitment of Lck into DRM

During physiological T cell stimulation by APCs, a major T cell membrane rearrangement is known to occur, leading to the organization of supramolecular activation clusters at the IS (28, 29). A possible role for the synapse is the generation of membrane compartments where signaling might be propagated (29). CD28 is recruited to the central cluster of the IS and is colocalized with the engaged TCR (30). It has been shown that CD28 triggering leads to higher and more stable tyrosine phosphorylation of several substrates and higher consumption of Lck while not promoting TCR engagement, and it was speculated that the higher Lck consumption was due to the CD28-mediated recruitment of lipid rafts (and therefore Lck) to the site of TCR triggering (20). However, using T cells from CD4- or CD28-deficient mice, it was suggested that Lck is recruited to the IS by CD4, whereas CD28 potentiates Lck autophosphorylation (23). We have previously shown that T cell stimulation by SEE-pulsed B cells induces recruitment of Lck into

DRM and that this is not mediated by TCR/CD3 engagement, because no enrichment of Lck in DRM can be induced by anti-CD3 Ab (31). Therefore, we asked whether CD4 or CD28 stimulation might be responsible for the Lck enrichment in DRM observed upon T cell activation.

PB T cells, stimulated or not with anti-CD28 or anti-CD4 Abs, were lysed in 0.2% Triton X-100 buffer on ice, and the lysates were subjected to discontinuous sucrose density gradient centrifugation. Individual light or heavy fractions were collected and subjected to SDS-PAGE and anti-Lck immunoblotting. CD28 engagement induced recruitment of Lck in the DRM, indicating a more stable association of Lck with lipid rafts (Fig. 1). In contrast, CD4 engagement did not modify Lck distribution among raft and nonraft fractions (Fig. 1). The recruitment of Lck into DRM after CD28 stimulation is not due to modifications of the plasma membrane merely induced by Ab cross-linking, as shown by the unmodified absence of CD3ϵ in DRM (Fig. 1).

CD28 stimulation induces Lck recruitment to the IS

In resting human PB T cells, TCR plus CD28 stimulation induces recruitment of raft markers to the TCR engagement site (20). We speculated that the stable association of Lck with lipid rafts induced by CD28 stimulation might be paralleled by a CD28- and lipid raft-mediated recruitment of Lck to the IS. Thus, we analyzed by confocal microscopy the distribution of Lck and lipid rafts in T lymphocytes incubated with bacterial superantigen (SAG)-pulsed 5-3.1 or 5-3.1/B7 cells. We visualized lipid rafts at the cytoplasmic leaflet of the plasma membrane by expressing MyrPalm-mCFP in resting PB T cells and in the Jurkat T cell line (5). In addition to the specific raft targeting, MyrPalm-mCFP is totally irrelevant for signaling and is thus not expected to interact with endogenous proteins to any significant extent (32). We found that Lck and MyrPalm-mCFP accumulated and colocalized at the IS only when T lymphocytes were incubated with 5-3.1/B7 cells, that is, only when CD28 and TCR were coengaged (Fig. 2, *A* and *B*). To exclude the possibility that the use of detergents might affect the analysis, the same experiments were performed in fixed, but not permeabilized, conjugates, and the recruitment of MyrPalm-mCFP was analyzed (Fig. 2*C*). The results obtained with or without detergent were very similar (Fig. 2, *A–C*). It is important to notice that CD4 was equally recruited to the IS with SAG-pulsed 5-3.1 or 5-3.1/B7 cells, indicating that in these experimental conditions it plays no major role in Lck as well as in lipid rafts mobilization (Fig. 2*D*). As expected, CD28-mediated Lck and MyrPalm-mCFP recruitment at the IS was inhibited in MβCD-treated cells (not shown).

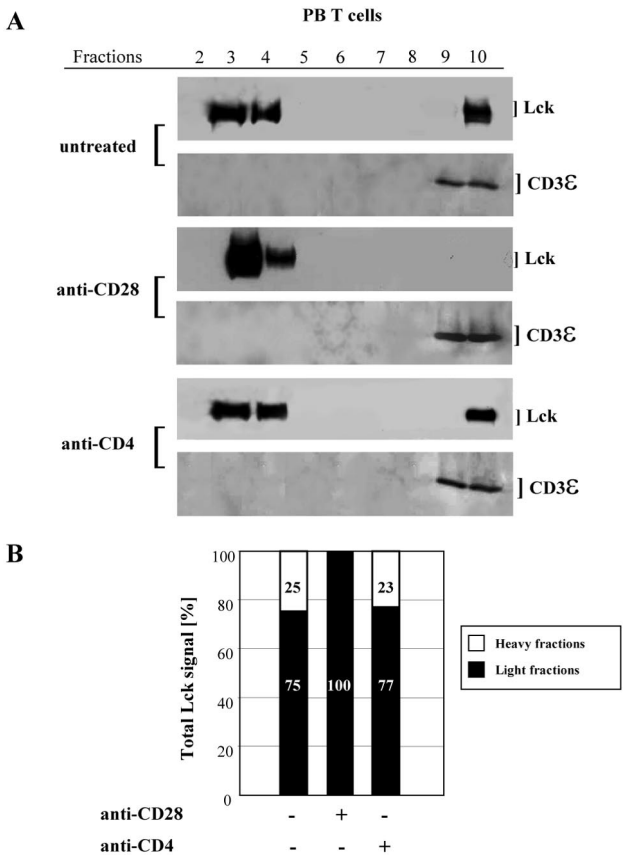

FIGURE 1. CD28, but not CD4, induces recruitment of Lck into DRM. *A*, PB CD4$^+$ T cells were left unstimulated or were stimulated with anti-CD28 or anti-CD4 Abs as described in *Materials and Methods*. Membrane fractions were separated, and samples were analyzed by Western blot using anti-Lck or anti-CD3ϵ Abs. *B*, Histograms show densitometric values of Lck subcellular distribution. Numbers represent the proportion of total Lck detected in raft (light) and nonraft (heavy) fractions.

Vav-1 is necessary for Lck recruitment to the IS

CD28 uses Vav-1 to integrate and amplify TCR-delivered signals. Vav-1 is a guanine nucleotide exchange factor for the Rho/Rac family of protein G and is involved in the activation of Rac-1 and Cdc42 (33). Vav-1 is strongly tyrosine phosphorylated upon CD28 stimulation (34, 35). This event induces the up-regulation of Vav-1 exchange activity for Rac-1 (36) and appears to be important in regulating the Vav-1 capacity to participate in the activation of several TCR- and CD28-mediated signaling pathways (37). In particular, Vav-1 is a key regulator of the actin cytoskeleton rearrangements that are necessary for the accumulation of signaling molecules and lipid rafts at the APC/T cell interface (38, 39). Thus, if Lck is recruited to the IS through CD28 and lipid rafts, this kinase should not be mobilized in T cells lacking Vav-1 activity. We stimulated the Vav-1-deficient cell line, J.Vav1, with SEE-pulsed 5-3.1/B7 cells, and in agreement with our hypothesis, we found that Lck did not accumulate at the IS and remained evenly distributed at the T cell membrane (Fig. 3). The capacity of CD28 to induce Lck accumulation at the contact region between triggered T cells and APC was completely restored when Vav-1 was re-expressed (Fig. 3).

The CD28 COOH-terminal PxxPP motif is necessary for Lck recruitment to the IS

Lck and Fyn regulate CD28-mediated Vav-1 tyrosine phosphorylation and activation (35). Three tyrosines and two PxxP motifs in the cytoplasmic domain of CD28 play a critical role in CD28 signaling (40). In particular, the COOH-terminal PxxPP motif has been reported to bind the SH3 domain of Lck and to be required for T cell costimulation (40). We have evidence that this motif is responsible for the CD28-induced recruitment of lipid rafts to the IS (5) and therefore we speculated that this region may be involved in the regulation of accumulation of Lck at the contact region between triggered T cells and APC. To test this hypothesis, we used a Jurkat cell line (CH7C17) deficient for CD28 expression reconstituted with human CD28 wild type (CD28WT) or with a CD28 mutant in the COOH-terminal PxxPP (CD28-3A), generated by substituting the prolines in positions 208, 211, and 212 with alanines. We found that CD28-3A cells were unable to redistribute Lck (as well as lipid rafts) upon stimulation with SEE-pulsed 5-3.1/B7 cells, indicating that the COOH-terminal PxxPP motif is essential for CD28-mediated Lck accumulation at the IS (Fig. 4). When the CD28WT was expressed in CH7C17 Jurkat cells, Lck

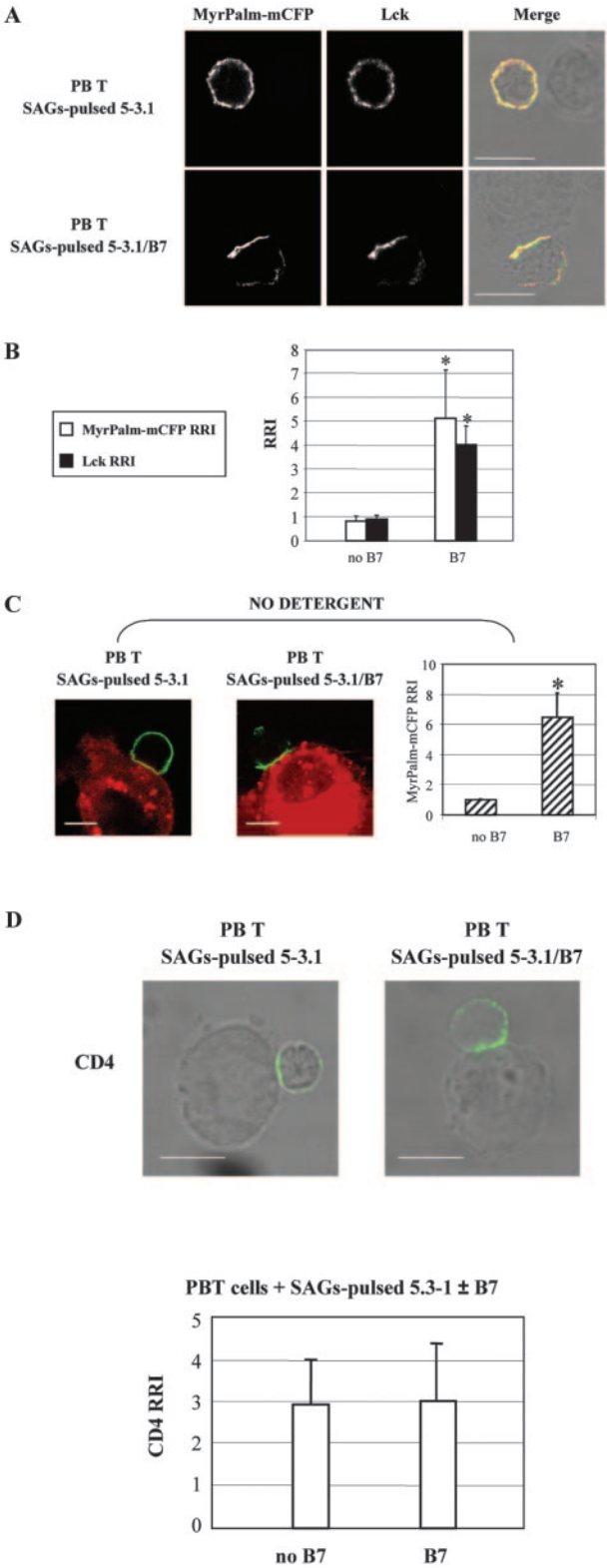

FIGURE 2. CD28 induces recruitment of Lck to the IS. *A*, PB CD4$^+$ T cells were transfected with MyrPalm-mCFP and incubated with SAG-pulsed 5-3.1 cells or with SAG-pulsed 5-3.1/B7 cells for 20 min, then fixed and stained with anti-Lck Abs, followed by FITC-conjugated anti-Ig Abs. The mCFP was color-coded red, anti-Ig-FITC was green, and yellow represents the superimposition of green and red. Confocal images were taken with identical settings. Representative data from one of four independent experiments are shown. Bar = 10 μm. *B*, Quantitative analysis of MyrPalm-mCFP and Lck accumulation at the IS of PB CD4$^+$ T cells with SAG-pulsed 5-3.1 or 5-3.1/B7 cells. The relative recruitment index (RRI) was calculated as described in *Materials and Methods* and represents the mean ± SD of 30 cells from four independent experiments. ∗, $p < 0.005$ compared with 5-3.1. *C*, PB CD4$^+$ T cells were transfected with MyrPalm-mCFP and incubated with SAG-pulsed 5-3.1 cells or with SAG-pulsed 5-3.1/B7 cells for 20 min, then fixed and directly analyzed (no detergent) as described in *A* and *B*. *D*, PB CD4$^+$ T cells were incubated with SAG-pulsed 5-3.1 or 5-3.1/B7 cells for 20 min, then fixed and stained with anti-CD4 Abs, followed by FITC-conjugated anti-Ig Abs. Confocal images were taken with identical settings. Representative data from one of three experiments are shown. Bar = 10 μm. The RRI of CD4 was calculated as described in *Materials and Methods* and represents the mean ± SD of 30 cells from three independent experiments.

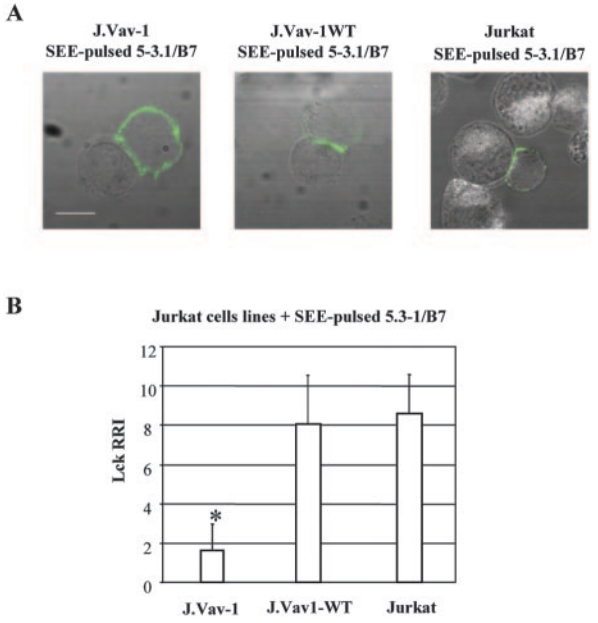

FIGURE 3. Lck accumulation at the IS requires Vav-1. *A*, J.Vav1, J.Vav1WT, or Jurkat cells were incubated with SEE-pulsed 5-3.1/B7 cells for 20 min, fixed, and stained with anti-Lck Abs, followed by FITC-conjugated anti-Ig Abs. Confocal images were taken with identical settings. Representative data from one of three experiments are shown. Bar = 10 μm. *B*, Quantitative analysis of Lck accumulation at the IS of J.Vav1, J.Vav1WT, or Jurkat cells with SEE-pulsed 5-3.1/B7 cells. The relative recruitment index (RRI) was calculated as described in *Materials and Methods* and represents the mean ± SD of 30 cells from three independent experiments. ∗, $p < 0.005$ compared with J.Vav1WT or Jurkat cells.

(and lipid rafts) accumulated at the IS as efficiently as in CD28$^+$ Jurkat cells (Fig. 4).

Discussion

We found that after CD28 engagement, Lck is recruited into CD28-signaling rafts and directed to the IS by a process requiring the CD28 COOH-terminal PxxPP motif and Vav-1. These results are in agreement with previous experiments showing that in human resting T cells, CD28 is able to induce raft clustering at the site of TCR engagement by Abs (20). Using Ab-coated beads and Jurkat T cells, it was suggested that the recruitment of rafts to the TCR contact site is not selective, but is a consequence of membrane ruffling (25). Indeed, in Jurkat cells stimulated with anti-CD3- plus anti-CD28-coated beads, we have never observed recruitment of raft molecules, whereas the recruitment was evident when T cells were stimulated by APC. The possibility that we are simply looking at membrane ruffling is ruled out by at least two findings: 1) in the same experimental conditions, not all the markers tested were

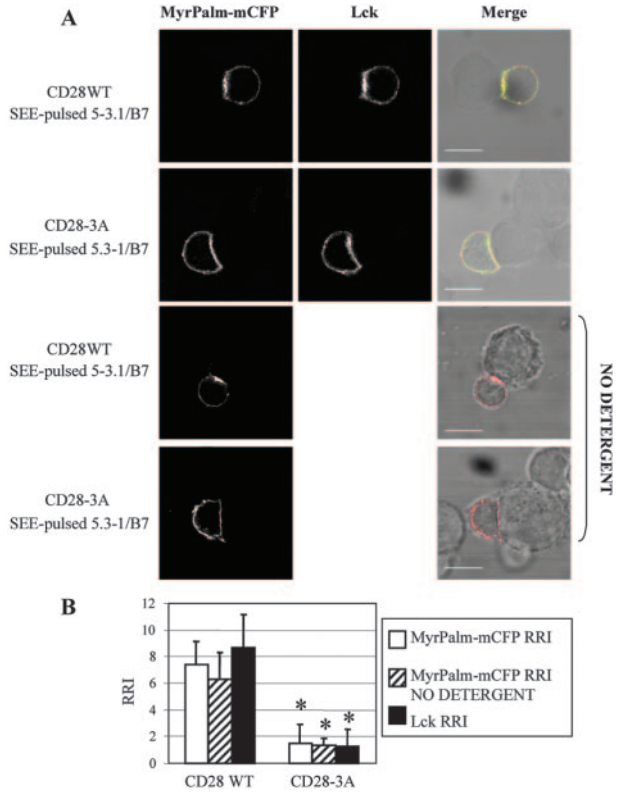

FIGURE 4. Lck accumulation at the IS requires the CD28 COOH-terminal PxxPP motif. *A*, CD28WT or CD28-3A Jurkat cells were transfected with MyrPalm-mCFP, then incubated with SEE-pulsed 5-3.1/B7 cells for 20 min, fixed, and either directly analyzed (no detergent) or stained with anti-Lck Abs, followed by FITC-conjugated anti-Ig Abs. mCFP was color-coded red, anti-Ig-FITC was green, and yellow represents the superimposition of green and red. Confocal images were taken with identical settings. Representative data from one of three experiments are shown. Bar = 10 μm. *B*, Quantitative analysis of Lck and MyrPalm-mCFP accumulation at the IS. The relative recruitment index (RRI) was calculated as described in *Materials and Methods* and represents the mean ± SD of at least 30 cells from three independent experiments. *, $p < 0.005$ compared with CD28WT Jurkat cells. In parallel experiments, the CD71 RRI was 0.9 ± 0.2.

accumulated (i.e., CD4 and Lck); and 2) molecules present only in conventional membranes did not accumulate at the IS (i.e., CD71 and LGFPGT46; see *Materials and Methods*). Therefore, the accumulation of molecules at the IS is a highly selective process that cannot be explained by membrane ruffling.

Using T cells from CD4- or CD28-deficient mice, it has been shown that Lck is recruited to the IS by CD4-MHC class II interaction, whereas CD28 potentiates Lck autophosphorylation (23). However, in human T cells, CD4 is recruited to and down-regulated with the triggered TCR as a consequence of binding of the CD4-associated Lck to ZAP-70 and independently of CD4-MHC class II interaction (41). Thus, CD4 associates the TCR even in T cells stimulated by anti-CD3 Abs, indicating that the coreceptor is recruited to the site of TCR engagement because of Lck and not vice versa (41). Our data indicate that CD28, and not CD4, mediates recruitment of Lck to the IS. Indeed, Lck was significantly accumulated at the IS only when T cells were stimulated with APC expressing the CD28 ligand and regardless of CD4 recruitment. Moreover, CD28, but not CD4 or CD3, stimulation induced accumulation of the kinase in DRM (31). The different results obtained by Shaw and coworkers (24) might be due to the use of T cells from $CD28^{-/-}$ mice, in which the accumulation of lipid rafts appears to be independent of CD28. Considering that CD28-deficient transgenic T cells develop normally and show only moderately impaired activation parameters (23) and that human naive T cells are, in contrast, highly dependent on CD28 signaling for activation, it would be difficult to compare the two systems. In contrast, it is possible that CD28 is not the only costimulatory molecule capable of recruiting lipid rafts to the IS and that the main difference in the two experimental systems is represented by the APCs used.

Vav-1 is considered one of the main effector molecules of CD28. Although TCR is able to activate Vav-1, the most prominent role in Vav-1 activation is played by CD28. Indeed, when compared with TCR triggering, CD28 stimulation induces a stronger and persistent tyrosine phosphorylation of Vav-1 (37, 42). Moreover, Vav-1 regulates CD28 activities as independent signaling unit (7, 8). Vav-1 is a key regulator of the actin cytoskeleton rearrangements that are necessary for accumulation of signaling molecules at the APC/T cell interface (38, 39), and Vav-1 deficient mice show specific defects in actin polymerization and T cell activation (43, 44). Altman and coworkers (39) have shown that Vav-1 is necessary to regulate lipid raft clustering and polarization at the IS, suggesting that actin cytoskeleton reorganization is required for stable lipid raft clustering. In agreement with these data and with our hypothesis that Lck is recruited to the IS through CD28 and lipid rafts, we found that in Jurkat cells lacking Vav-1 and stimulated with $B7^+$ APCs, Lck does not accumulate at the IS.

Shaw and coworkers (40) have shown that C-terminal proline residues of CD28 are required for Lck binding as well as for CD28-mediated T cell costimulation. In this paper, we identified the proline residues of CD28 as the region responsible for CD28-mediated Lck recruitment to the IS. Taken together, the results obtained in the two studies demonstrate that the physical interaction of CD28 with Lck is relevant for CD28 signaling leading to lipid raft mobilization, Lck enrichment at the IS, and T cell costimulation.

Together, our results demonstrate that CD28 and lipid rafts cooperate in generating, at the IS, a kinase-enriched environment where TCR signal transduction can be amplified and sustained.

What is the mechanism used by CD28 to mobilize and recruit lipid rafts? The fact that CD28 signaling and Vav-1 are required suggests that an active process, probably involving actin cytoskeleton, is responsible for raft targeting to the IS. Recently, it has been shown that activation-induced polarized recycling targets TCR to the IS (45). We hypothesize that a similar process, together with changes in the dynamics of plasma membrane rafts, might be responsible for the observed accumulation of raft markers at the site of TCR and CD28 triggering. The identification of the CD28 motif responsible for raft recruitment to the IS will allow us to start a detailed investigation of the mechanisms involved in raft organization and mobilization in T cells.

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