Src Homology 2-Domain Containing Leukocyte-Specific Phosphoprotein of 76 kDa Is Mandatory for TCR-Mediated Inside-Out Signaling, but Dispensable for CXCR4-Mediated LFA-1 Activation, Adhesion, and Migration of T Cells

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J Immunol published online 7 October 2009
http://www.jimmunol.org/content/early/2009/10/07/jimmunol.0900649

Supplementary Material
http://www.jimmunol.org/content/suppl/2009/10/06/jimmunol.0900649.D1

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SRC HOMOLOGY 2-DOMAIN CONTAINING LEUKOCYTE-SPECIFIC PHOSPHOPROTEIN OF 76 kDa Is Mandatory for TCR-Mediated Inside-Out Signaling, but Disposable for CXCR4-Mediated LFA-1 Activation, Adhesion, and Migration of T Cells

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Engagement of the TCR or of chemokine receptors such as CXCR4 induces adhesion and migration of T cells via so-called inside-out signaling pathways. The molecular processes underlying inside-out signaling events are as yet not completely understood. In this study, we show that TCR- and CXCR4-mediated activation of integrins critically depends on the membrane recruitment of the adhesion- and degranulation-promoting adapter protein (ADAP)/Src kinase-associated phosphoprotein of 55 kDa (SKAP55)/Rap1-interacting adapter protein (RIAM)/Rap1 module. We further demonstrate that the Src homology 2 domain containing leukocyte-specific phosphoprotein of 76 kDa (SLP76) is crucial for TCR-mediated inside-out signaling and T cell/APC interaction. Besides facilitating membrane recruitment of ADAP, SKAP55, and RIAM, SLP76 regulates TCR-mediated inside-out signaling by controlling the activation of Rap1 as well as Rac-mediated actin polymerization. Surprisingly, however, SLP76 is not mandatory for CXCR4-mediated inside-out signaling. Indeed, both CXCR4-induced T cell adhesion and migration are not affected by loss of SLP76. Moreover, after CXCR4 stimulation, the ADAP/SKAP55/RIAM/Rap1 module is recruited to the plasma membrane independently of SLP76. Collectively, our data indicate a differential requirement for SLP76 in TCR- vs CXCR4-mediated inside-out signaling pathways regulating T cell adhesion and migration. The Journal of Immunology, 2009, 183: 0000–0000.

stimulation of T cells through the TCR activates a whole plethora of signaling pathways that collectively control activation, proliferation, and differentiation of T cells. One immediate consequence of TCR engagement is the formation of a multicomponent signaling complex close to the plasma membrane consisting of the Src homology 2-domain containing leukocyte-specific phosphoprotein of 76 kDa (SLP76),⁴ the small adapter protein Gads, and the transmembrane adapter protein linker for activation of T cells (LAT) (1). In response to TCR engagement, LAT becomes phosphorylated on several tyrosine residues by the protein tyrosine kinase ZAP70 (1). This phosphorylation leads to recruitment of Gads. Through its constitutive association with Gads, SLP76 is also recruited to phosphorylated LAT (2). In the following, several other effector molecules assemble with the LAT/Gads/SLP76-signaling platform. These include phospholipase Cγ1 (PLCγ1), the nucleotide exchange factor Vav1, and the Tec family kinase IL-2-inducible T cell kinase (Itk) (1). Together these molecules coordinate TCR-mediated rises in intracellular calcium, up-regulation of CD69, and the activation of the Ras/ERK1/2 signaling pathway (1, 3).

The importance of SLP76 for TCR-mediated signaling events has been demonstrated in SLP76-deficient mice as well as in the SLP76-deficient Jurkat T cell line J14. Thus, loss of SLP76 in mice leads to a complete block at the double-negative 3 stage of thymic development and results in an almost complete loss of mature T cells (1). Moreover, loss of SLP76 in Jurkat T cells induces a complete failure of the TCR to induce rises in intracellular calcium, activation of the Ras/Raf/MAPK/ERK1/2 pathway, and up-regulation of CD69 expression (3).

Besides inducing the above-mentioned signaling events, TCR stimulation also leads to T cell adhesion, a process that is critical for the interaction between T cells and APCs. In T cells, the β2 integrin LFA-1 (αβ2) mediates adhesive events with APCs through binding to its ligand ICAM-1. LFA-1/ICAM-1 interactions are critical for T cell activation because they stabilize the interaction of T cells with APCs and the formation of the immunological synapse (IS) (4, 5).
Resting T cells are not adhesive because LFA-1 is presented in a closed, inactive conformation. Upon triggering of the TCR or of chemokine receptors (see below), a conformational change is induced within LFA-1 that augments its affinity for ICAM-1. In addition, clustering of LFA-1 molecules on the surface of T cells enhances avidity for ICAM-1 binding. The molecular events leading to integrin activation have collectively been termed inside-out signaling (6).

Gain-of-function and loss-of-function studies have demonstrated that additional signaling proteins are involved in TCR-mediated integrin activation. These include talin (6, 7), the Wiskott-Aldrich syndrome-Verprolin-homologous protein WAVE2 (8, 9), Rap1 (6, 10, 11), and its downstream targets regulator of adhesion and cell polarization enriched in lymphoid tissues or Rap1-interacting adapter protein (RIAM) (12–14), adhesion- and degragation-promoting adapter protein (ADAP) (15, 16), and Src kinase-associated phosphoprotein of 55 kDa (SKAP55) (17, 18). Moreover, components of the LAT/Gads/SLP76 signaling platform are also critically involved in TCR-mediated activation of LFA-1. These include PLCγ1, Vav1, Itk, and ADAP (13, 19, 20). How exactly these molecules orchestrate TCR-mediated activation of integrins is not completely understood. However, we have recently shown that the formation of a signaling module consisting of ADAP/SKAP55 complex together with RIAM is required for plasma membrane targeting of Rap1 in response to TCR stimulation, and therefore for TCR-mediated activation of integrins (17, 21). In line with these data is the observation that ADAP- and SKAP55-deficient mouse T cells show a severe defect in integrin activation in response to TCR-mediated stimuli (15, 16, 18).

Currently, it is proposed that following T cell activation, tyrosine-phosphorylated ADAP binds to the Src homology 2 domain of SLP76, thereby leading to membrane recruitment of the ADAP/ SKAP55/RIAM module and integrin activation. In line with this idea is the observation that disruption of the interaction between SLP76 and Gads blocks TCR-mediated adhesion to ICAM-1 (22). Furthermore, it was shown that mutation of those tyrosine residues within ADAP, which are believed to mediate the interaction between ADAP and SLP76, blocks TCR-mediated activation of integrin (23). Thus, it appears as if SLP76 would also play a major role during TCR-mediated activation of integrins by facilitating membrane targeting of the ADAP/ SKAP55/RIAM module. However, to date, an inducible interaction between endogenously expressed ADAP/SKAP55/RIAM module and SLP76 in response to TCR engagement has not been demonstrated biochemically. Hence, the molecular signaling events underlying the function of SLP76 for TCR-mediated integrin activation are not completely elucidated.

Importantly, integrins not only become activated after stimulation of the TCR, but also after triggering of chemokine receptors, such as CXCR4. Signaling via CXCR4 is induced by stromal cell-derived factor-1α (or CXCL12) and induces affinity and avidity regulation of LFA-1 (6). This process is important for firm T cell adhesion, T cell polarization, chemokinesis, and chemotaxis (24). Molecules involved in CXCR4-mediated integrin activation and/or chemotaxis are talin (25), Rap1 (6, 26, 27), and its downstream targets regulator of adhesion and cell polarization enriched in lymphoid tissues and Mst1 (28–30), Itk (20), Vav1 (20, 31, 32), Rac (31), and members of the Wiskott-Aldrich syndrome protein family (33, 34). Moreover, we and others have shown that overexpression of ADAP enhances chemotaxis of T cells in response to CXCL12 (35, 36). These findings suggest an important role of ADAP for CXCR4-induced migration of T cells. However, the molecular basis of how ADAP is integrated into CXCR4 signaling is to date unclear. Based on its central role in TCR-mediated signaling processes, SLP76 would be an attractive candidate that could facilitate membrane targeting of ADAP after CXCR4 stimulation. Indeed, it was shown recently that SLP76 regulates CXCR4-induced Ca²⁺ flux and ERK1/2 phosphorylation (31). Conversely, we had demonstrated that disruption of the SLP76/Gads association, albeit impairing TCR-mediated signaling processes, does not affect CXCR4-induced Ca²⁺ flux and chemotaxis (22). Thus, it is unclear whether SLP76 is also important for CXCR4-mediated activation of integrins and chemotaxis.

In this study, we have addressed critical questions regarding the function of SLP76 during TCR vs CXCR4-mediated signaling. We show that SLP76 is indeed a critical regulator of TCR-mediated inside-out signaling events in T cells, and we demonstrate that SLP76 is mandatory for induction of TCR-mediated adhesion, affinity/avidity regulation of LFA-1, and the interaction between T cells and B cells. Furthermore, we show that SLP76 is required for TCR-induced Rap1 activation, Rac-mediated actin dynamics, and recruitment of both talin and the ADAP/SKAP55/RIAM/Rap1 module to the plasma membrane and to the IS. Surprisingly, however, SLP76 is not mandatory for CXCR4-mediated activation of LFA-1. In addition, SLP76 is dispensable for adhesion and migration of T cells in response to CXCL12. Finally, we demonstrate that the ADAP/SKAP55/RIAM/Rap1 module is crucial for T cell adhesion and migration in response to CXCR4 triggering, but is recruited to the plasma membrane independently of SLP76. Our findings show that SLP76 acts as a key player during TCR-mediated inside-out signaling, whereas the adapter protein appears to be dispensable for chemokine-dependent processes that regulate adhesion and migration of T cells.
Signaling Technology), anti-Rap1 rabbit serum (Santa Cruz Biotechnology), anti-Rap1 mAb and anti-Rac mAb (both from BD Biosciences), and anti-talin mAb (clone 8D4; Sigma-Aldrich). The following Abs were used for phospho-epitope staining by FACS: anti-phospho-ZAP70 (pY319)-Alexa Fluor 647, anti-phospho-PLCγ1 (pY783)-Alexa Fluor 647 (both from BD Biosciences), and anti-phospho-ERK1/2 (pT202, pY204; clone E10; Cell Signaling Technology). Streptavidin, HRP-labeled secondary Abs, and FITC-, Cy3-, APC-, and Cy5-conjugated secondary Abs were purchased from Dianova.

RNA interference (RNAi) of SLP76, ADAP, SKAP55, or RIAM and cDNA constructs

For small hairpin RNA (shRNA) of SLP76, the following oligonucleotides, shC (CCAGGATAGTGGATCAA; Renilla) and shSLP76 (CGAAGAGGAGGAGACCT; shSLP76), were cloned into pSuper (provided by T. Seufferlein (Universitätshospital und Poliklinik für Innere Medizin I, Halle, Germany) and pCMS3-EGFP vector (provided by D. Billadeau (Mayo Clinic, Rochester, MN). For shRNA of ADAP and RIAM, the previously used pSUPER-EGFP vector (Santa Cruz Biotechnology) was cloned into the pCMS3 vector (12, 42). The pCMS-EGFP construct for silencing of SLP75 has been described (17). The pGEX-PAK-PBD was provided by T. Seufferlein. The pGEX RaGDS-RBD was provided by J. Bos (University Medical Center, Utrecht, The Netherlands), and the pmCherry-C1 vector was purchased from BD Clontech.

Cell culture and transfection

Jurkat T cells (ATCC), B cells (Raji; ATCC), and SLP76-deficient/reconstituted Jurkat T cells (J14, J14-76-11, and J14-76-18) (3) were maintained in RPMI 1640 medium supplemented with 10% FBS (PAN) and stable t-glutamine at 37°C with 5% CO2. Jurkat T cells (2 × 10⁴) were transfected by electroporation, as previously described (17). Transfection with the pCMS3-EGFP vector into Jurkat T cells consistently yielded in an average of >80% GFP-expressing cell population. Primary human T cells were prepared from healthy donors by standard separation methods using AutoMACS (Miltenyi Biotec) maintained in RPMI 1640 medium containing 10% FBS, stable t-glutamine, and 1000 U/ml penicillin/streptomycin. Approval for these studies was obtained from the Ethics Committee of the Medical Faculty at the Otto-von-Guericke University. Informed consent was obtained in accordance with the Declaration of Helsinki. For electroporation of small interfering RNA (siRNA), human peripheral T cells (8 × 10⁴) were washed in PBS containing Ca²⁺/Mg²⁺ and resuspended in 200 µl of Opti-MEM (Invitrogen). siRNA smart pool against SLP76, ADAP (Dharmacon MEM (Invitrogen). siRNA smart pool against SLP76, ADAP (Dharmacon), and cDNA constructs

Conjugate formation, adhesion, migration, and motility assays

Conjugate assays were performed, as described (21). Briefly, superantigen (SA) mixture of staphylococcal enterotoxin B-, D-, and E-pulsed and Daudi-labeled Raji B cells were incubated with an equal number of Jurkat T cells or CFSE-loaded human T cells for 30 min at 37°C. Non-specific aggregates were disrupted; cells were fixed with 1% PFA, and then analyzed by flow cytometry. The percentage of conjugates was defined as the number of double-positive events in the upper right quadrant. Adhesion assays were performed, as previously described (17). Briefly, Jurkat T cells or peripheral human T cells were stimulated with OKT3, PMA, or MntCl, for 30 min at 37°C before adhesion on Fc-ICAM-1-coated dishes. The bound total or GFP-expressing cell fraction was determined by counting four independent fields by microscopy using an ocular counting reticule. To assess CXCR4-mediated adhesion, peripheral human T cells were incubated for 10 min at 37°C on Fc-ICAM-1-coated dishes coimmobilized with or without CXCL12; subsequently, nonbound cells were removed by washing with HBSS and bound cells were counted, as described above. Chemotaxis assays were performed, as previously described, using Transwells (Costar) coated with fibronectin (35). After 2 h, the number of migrated cells into the lower chamber was counted and the percentage of GFP-expressing Jurkat T cells was determined by flow cytometry. For live cell imaging of either random or CXCL12-induced motility of T cells on Fc-ICAM-1, self-constructed imaging chambers (46) were coated with Fc-ICAM-1 in PBS at 4°C overnight. Immediately before imaging, cells were left untreated or stimulated with CXCL12 (100 ng/ml), and imaging was performed on a CellIR imaging workstation (Olympus) using an upright microscope stage (BX61) with a ×20 lens. An automated X-Y-Z stage, at least two optical fields were chosen for each culture condition. Images were taken every 15–60 s for 2 h. At least 30 cells per observation field were analyzed. Tracking analysis of migrating cells to determine the velocity was done by computer-assisted cell tracking using the Software CellTracker (46).

Immunofluorescence microscopy

For B cell/T cell conjugates, SA- and Blue-7-amino-4-chloromethylcoumarin-loaded Raji B cells were incubated for 30 min at 37°C with human T cells on poly-l-lysine)-coated coverslips and fixed with 3.5% PFA in PBS for 10 min. Cells were permeabilized with 0.1% Triton X-100 in PBS, blocked with 5% horse serum in PBS, and incubated with the indicated Abs or phalloidin. Coverslips were mounted in Mowiol 488 and imaged with a Leica TCS SP2 laser scanning confocal microscope systems using a plan apochromatic oil emerged ×63 objective (NA 1.4). Image constructions of images were performed in COREL Photopaint. For quantification of recruitment of proteins and F-actin at the contact zone or at the uropod, line scans (1 µm) were obtained. Fluorescence intensity was plotted as function of distance along this line, and the integrated areas under the curves representing the fluorescence intensity were calculated as ratio between the immunological synapse vs the uropod. Thirty conjugates were analyzed per experiment. To assess TCR-induced clustering of LFA-1, Jurkat T cells were incubated with biotinylated anti-UCHT-1 in the presence of streptavidin at 4°C. After washing, the cells were stimulated at 37°C for 30 min on poly-l-lysine)-coated slides, fixed with 3.5% PFA in PBS, and then blocked with 5% horse serum in PBS. The cells were stained

5 The online version of this article contains supplemental material.
with FITC-conjugated anti-MEM48 and imaged, as described above. For each experiment, a minimum of at least 40 cells with LFA-1 polarized to one side of the cell was regarded as polarized, whereas those cells showing equal distribution of LFA-1 were considered not to be polarized. The percentage of polarized cells in each field was determined.

**Results**

**Loss of SLP-76 impairs TCR-mediated activation of LFA-1 and T cell-APC conjugation**

By using a small peptide that disrupts the constitutive interaction between SLP76 and Gads, we recently provided evidence that formation of the LAT/Gads/SLP76 signaling platform at the plasma membrane is required for TCR-mediated integrin activation (22). However, we formally could not exclude the possibility that the functional effects exerted by the Gads-binding fragment were not due to targeting the SLP76/Gads complex, but rather to disruption of a distinct signaling pathway that regulates integrin activation in response to TCR stimulation. To assess this point more specifically, we reduced the expression of SLP76 in primary human T cells by RNAi. As shown in Fig. 1, SLP76 siRNA treatment lowered the expression levels of endogenous SLP76 up to 80%, whereas the expression levels of ADAP, SKAP55, and RIAM remained unaffected.
Previously, it had been reported that SLP76 is required for phosphorylation of PLCγ1, activation of ERK1/2, as well as expression of CD69 upon TCR stimulation. To first address whether loss of SLP76 interferes with these SLP76-mediated functions also in primary human T cells, we analyzed the ability of SLP76-deficient human T cells to activate PLCγ1 and ERK1/2 in response to TCR stimulation by Western blotting and to up-regulate CD69 expression by flow cytometry. As shown in Fig. 1B, loss of SLP76 strongly attenuated TCR-mediated phosphorylation of PLCγ1 at Y783, activation of ERK1/2, and up-regulation of CD69. As expected, more proximal signaling events such as the phosphorylation of ZAP70 at Y192 or tyrosine phosphorylation of LAT (either global or at Y177) were not affected in the absence of SLP76 (Fig. 1C). Note that similar data were obtained, when we suppressed the expression of SLP76 by shRNA in Jurkat T cells (supplemental Fig. 2). Thus, with regard to well-established SLP76-mediated signaling processes, SLP76-deficient primary human T cells behave like their corresponding Jurkat T cell counterparts (3).

Using the siRNA approach, we next analyzed the capability of T cells to adhere to ICAM-1-coated dishes in response to various stimuli. As shown in Fig. 1D and supplemental Fig. 3A, control transfected human T cells and Jurkat T cells readily adhered to ICAM-1 upon TCR or PMA treatment. In contrast, loss of SLP76 substantially attenuated both PMA- and TCR-induced adhesion to ICAM-1 (left panel), whereas the cells showed no defect in their adhesiveness in response to Mn2+ (middle panel). The defect in TCR- or PMA-induced adhesion to ICAM-1 was not due to an altered expression of the β2 integrin (or the TCR) as determined by flow cytometry (Fig. 1E and supplemental Fig. 3B).

Because the interaction of LFA-1 with ICAM-1 is important for the establishment and maintenance of T cell/APC interactions (5), we next analyzed whether SLP76 is required for conjugate formation between human T cells and SA-loaded B cells. Fig. 1F shows that in contrast to control transfected T cells, loss of SLP76 substantially blunted conjugate formation. Similarly, SLP76low Jurkat T cells failed to interact with SA-loaded B cells (supplemental Fig. 3C). Collectively, the experiments shown in Fig. 1 indicate that SLP76 is mandatory for both TCR-mediated adhesion to ICAM-1 and conjugate formation.

**Loss of SLP76 attenuates TCR-mediated affinity/avidity regulation of LFA-1**

TCR-mediated inside-out signaling alters both LFA-1 affinity (conformation) and avidity (clustering) (6). To determine the role of SLP76 for LFA-1 affinity modulation, we assessed the ability of soluble Fc-ICAM-1 to bind to SLP76-proficient and SLP76-deficient T cells after TCR stimulation by means of flow cytometry. Fig. 2A shows that knockdown of SLP76 in human T cells abrogates binding of soluble Fc-ICAM-1 after TCR stimulation (for Jurkat T cells, please see supplemental Fig. 3D). In contrast, both transfecteds were able to bind similar amounts of Fc-ICAM-1 after treatment with Mg2+/EDTA, which directly induces the high-affinity conformation of LFA-1.

Next, we investigated avidity regulation of LFA-1 by analyzing clustering of CD18 (the β2 chain of LFA-1) to the IS in response to TCR stimulation. Fig. 2B shows that localization of CD18 to the IS occurred readily in SLP76-expressing T cells after incubation with SA-pulsed B cells, whereas it was severely impaired in SLP76low T cells. In line with these data, SLP76low Jurkat T cells also displayed no typical clustering of CD18 upon cross-linking of the TCR (supplemental Fig. 3E). Taken together, SLP76 is required for both affinity and avidity regulation of LFA-1 after stimulation of the TCR.

**TCR-mediated activation of Rac and actin dynamics require SLP76**

Reorganization of the actin cytoskeleton is required for TCR-mediated integrin activation and conjugate formation (33, 34), and the small GTPase Rac is a major regulator regulating this process. In T cells, activation of Rac is mediated via the nucleotide exchange factor Vav1, which is recruited to and activated by SLP76 upon T cell activation (32). Therefore, it seemed likely that loss of SLP76 also leads to alterations in actin dynamics upon TCR stimulation. As depicted in Fig. 3, this is indeed the case. TCR-mediated activation of Rac (Fig. 3A), formation of F-actin (Fig. 3B), as well as accumulation of F-actin at the IS (Fig. 3C, upper right panel) are severely impaired in SLP76-deficient T cells. Note that similar data were obtained when the F-actin content after TCR triggering was assessed in SLP76low Jurkat T cells (data not shown). Hence, loss of SLP76 induces a failure to activate Rac and a defect in F-actin remodeling upon TCR stimulation.

Because the scaffolding protein talin links newly synthesized F-actin branches to LFA-1 and, hence, stabilizes the position of LFA-1 in the IS (7, 47), we also investigated whether SLP76 is required for targeting of talin to the IS after TCR stimulation.
SLP76-DEPENDENT SIGNALING FOR LFA-1 ACTIVATION

Both the activation and the transport of Rap1 to the plasma membrane are known to regulate LFA-1 activation (6, 17, 48). Therefore, we next investigated whether loss of SLP76 affects TCR-mediated activation of Rap1. Fig. 4A demonstrates that control transfected cells exhibited strong activation of Rap1 within 2–10 min after TCR stimulation, whereas in SLP76-deficient Jurkat T cells, TCR-induced Rap1 was strongly suppressed. These results demonstrate that SLP76 is critically involved in Rap1 activation in response to TCR triggering.

We have recently shown that formation and membrane recruitment of a signaling module consisting of the cytosolic adapter proteins ADAP and SKAP55 and the Rap1 effector molecule RIAM (ADAP/SKAP55/RIAM module) regulate TCR-mediated LFA-1 activation through recruitment of Rap1 to the plasma membrane (17, 21). Moreover, an inducible association between SLP76 and ADAP following TCR stimulation had been suggested in several previous studies, but the question of whether SLP76 links the TCR via the ADAP/SKAP55/RIAM module to integrin activation has to date not been addressed directly. To clarify this point, we immunoprecipitated SLP76 from resting or TCR-stimulated T cells and subsequently analyzed the precipitates for coprecipitation of ADAP, SKAP55, RIAM, and Rap1 by means of Western blotting. Fig. 4B depicts that SLP76 inducibly associates with both the ADAP/SKAP55/RIAM complex and Rap1 upon TCR stimulation of Jurkat T cells (Fig. 4B, left panel) as well as of primary T cells (Fig. 4B, right panel). Analysis of SKAP55 immunoprecipitates that were prepared from either SLP76-proficient or SLP76-deficient T cells further revealed that Rap1 only associates with the ADAP/SKAP55/RIAM module if SLP76 is present (Fig. 4C). Because only active Rap1 can interact with RIAM (12), this finding is most likely due to the attenuated activation of Rap1 in the absence of SLP76 (see Fig. 4A).

The immunoprecipitation data shown in Fig. 4, B and C, were further substantiated by cellular subfractionation experiments that showed that TCR-mediated plasma membrane recruitment of ADAP, SKAP55, RIAM, and Rap1 (Fig. 4D) and, consequently, targeting of SKAP55, RIAM, and Rap1 to the IS (Fig. 4E) were strongly impaired in SLP76-deficient T cells. In summary, the data shown in Fig. 4 indicate that SLP76 is required for both activation and plasma membrane/IS targeting through the ADAP/SKAP55/RIAM/Rap1 module of Rap1 after TCR triggering.

SLP76 is dispensable for CXCR4-mediated adhesion to ICAM-1 and affinity regulation of LFA-1

Similar to the TCR, triggering of the chemokine receptor CXCR4 leads to activation of LFA-1 and T cell adhesion to ICAM-1 (6). Above we have shown that SLP76 is mandatory for TCR-mediated integrin activation (Figs. 1 and 2). Therefore, we were interested to investigate whether SLP76 is also required for CXCR4-mediated adhesion. To assess this point, we down-regulated SLP76 expression by siRNA and subsequently analyzed the capability of SLP76-deficient T cells to adhere to ICAM-1 in response to CXCL12 in an adhesion assay. Surprisingly, these experiments revealed that SLP76-deficient T cells were as capable to adhere to Fc-ICAM-1 in response to CXCL12 as their SLP76-proficient counterparts (Fig. 5A). Similarly, neither the binding of soluble Fc-ICAM-1 (Fig. 5B) nor of the conformation-sensitive anti-LFA-1 mAb KIM127 (data not shown) was altered in SLP76-deficient T cells. Hence, we conclude that, in contrast to TCR stimulation, the presence of SLP76 is dispensable for T cell adhesion and affinity regulation in response to CXCL12 stimulation.

Fig. 3C (lower right panel) shows that SLP76-deficient T cells also fail to target talin to the IS upon incubation with SA-pulsed B cells. Thus, SLP76 appears to also regulate LFA-1-dependent adhesion processes in activated T cells through its ability to recruit talin to the IS.

FIGURE 3. TCR-mediated actin remodeling, Rac activation, and recruitment of talin to the IS require the presence of SLP76. A, Jurkat T cells were transfected with either control vector (shC) or SLP76-suppression vector (shSLP76). After 48 h, cells were either left untreated or stimulated with anti-CD3 mAbs (TCR) for the indicated periods of time. GTP-loaded Rac was precipitated using the GST-PBD fusion protein. Precipitates and aliquots of whole-cell extracts were analyzed using the indicated Abs by means of Western blotting. One representative experiment of two is shown. B, Purified human T cells were transfected with either control siRNA (siC) or siRNA against SLP76 (siSLP76). After 72 h, cells were left untreated or stimulated with anti-CD3 mAbs (TCR) for 5 min and stained with FITC-coupled phalloidin. Reduction of SLP76 expression in human T cells was assessed by flow cytometry. Data are representative of three independent experiments. C, Human T cells transfected as described above were allowed to form conjugates with SA-loaded B cells for 30 min. Cells were fixed, permeabilized, and stained with TRITC-phalloidin (red) or talin (green). Fluorescence intensity of F-actin and talin at IS or uropod was quantified and calculated as ratio of intensity for the individual molecules at the IS vs the uropod. The knockdown efficiency of SLP76 was evaluated by flow cytometry. Data present the average results of three independent experiments.

Activation of Rap1 and localization of the SKAP55/RIAM module to the IS depend on SLP76

SLP76-dependent signaling for LFA-1 activation through its ability to recruit talin to the IS.
SLP76 is dispensable for CXCR4-mediated Rac activation, actin dynamics, and migration

Similar to TCR stimulation, remodeling of the actin cytoskeleton through Rac is crucial for inducing T cell adhesion, polarization, and chemotaxis following CXCR4 stimulation (33). Our above findings led us to ask whether the presence of SLP76 might be dispensable for Rac activation in response to CXCL12 triggering. As shown in Fig. 6A, SLP76low Jurkat T cells indeed display no obvious defect in CXCL12-mediated activation of Rac. Moreover, both SLP76high and SLP76low T cells revealed comparable levels of F-actin formation in response to CXCL12 (Fig. 6B). These data strongly suggest that SLP76 is indeed dispensable for signaling events that are critically involved in CXCR4-mediated activation of Rac and actin polymerization.

To prove whether expression of SLP76 is required for CXCR4-induced T cell chemokinesis, we determined the lateral locomotion of T cells by live cell imaging on Fc-ICAM-1-coated coverslips. As shown in Fig. 6C, both SLP76high and SLP76low T cells showed comparable basal and CXCL12-induced velocities. In contrast, and in line with previously published data (31), Vav1-deficient T cells displayed...
a strongly reduced motility upon CXCR4 triggering (Fig. 6C). It is also important to note that nearly all SPL76low T cells displayed a polarized phenotype upon chemokine stimulation, whereas the majority of Vav1-depleted T cells remained a round, nonpolarized shape (Fig. 6C). Fig. 6D further demonstrates that the absence of SPL76 does not alter CXCR4-induced chemotaxis of human primary T cells in response to a CXCL12 gradient in vitro. In summary, it appears as if SPL76 is not mandatory for migratory steps triggered by CXCL12.

The ADAP/SKAP55/RIAM module is recruited to the plasma membrane independently of SPL76

Upon T cell activation, the Gads/SLP76 complex is recruited to phosphorylated LAT, thereby coupling the TCR to the intracellular signaling machinery (2, 49). Given the above data, we next investigated whether the LAT/Gads/SLP76 signaling platform would be assembled after CXCR4 stimulation. To test this, we stimulated human T cells for various periods of time with CXCL12 and subsequently analyzed global tyrosine phosphorylation of LAT by Western blotting. In contrast to TCR stimulation, phosphorylation of LAT was only very weakly induced upon CXCR4 triggering (Fig. 7A). Similar results were obtained when the phosphorylation status of LAT at Y171 (which is important for Gads binding) was assessed (Fig. 7A). These data suggested that the Gads/SLP76 complex might not associate with LAT following CXCR4 triggering. To investigate this point in more detail, we immunoprecipitated the Gads/SLP76 complex from untreated, TCR-stimulated, or CXCR4-triggered T cells. Fig. 7B shows that an inducible interaction among Gads/SLP76, LAT, and Vav1 was readily detectable after TCR stimulation. In contrast, neither LAT nor Vav1 was found to be associated with the Gads/SLP76 complex in

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**FIGURE 5.** SLP76 is dispensable for CXCL12-mediated adhesion to ICAM-1, affinity maturation of LFA-1. A. Human T cells were transfected with control siRNA (siC) or siRNAs against SLP76 (siSLP76). Cells were left untreated or stimulated with immobilized CXCL12 (10 min) or anti-CD3 mAbs (TCR) for 30 min. Subsequently, cells were analyzed for their ability to adhere to Fc-ICAM-1. Suppression of SLP76 expression was assessed by flow cytometry, and the percentage of SLP76-expressing cells was calculated. Data represent the mean and SE of three independently performed experiments. B. Human T cells transfected as described in A were analyzed for their ability to bind soluble Fc-ICAM-1 upon anti-CD3 (TCR), CXCL12, or MgCl2/EGTA (positive control) treatment for 5 min. Suppression of SLP76 expression was evaluated by flow cytometry. One individual experiment of three is shown.

**FIGURE 6.** The presence of SLP76 is not required for Rac activation, actin polymerization, motility, and chemotaxis in response to CXCL12. A. Jurkat T cells were transfected with either control vector (shC) or SLP76-suppression vector (shSLP76). After 48 h, cells were either left untreated, or stimulated with CXCL12 or anti-CD3 mAbs (TCR) for the indicated time points. Activated Rac was precipitated using the GST-PBD fusion protein. Precipitates and aliquots of whole-cell extracts were analyzed by Western blotting using the indicated Abs. One representative experiment of three is shown. B. Human T cells were transfected with control siRNA (siC) or siRNA against SLP76 (siSLP76). After 72 h, cells were left untreated, stimulated with anti-CD3 mAbs (TCR, for 5 min) or CXCL12 for 1 min, and stained with FITC-coupled phalloidin. Reduction of SLP76 expression in human T cells was assessed by flow cytometry. Data are representative of three independent experiments. C. Human T cells were transfected with control siRNA (siC), siRNA against SLP76 (siSLP76), or siRNA against Vav1 (siVav1). After 72 h, motility of T cells in the absence or presence of CXCL12 was determined on Fc-ICAM-1-coated coverslips, as described in Materials and Methods. In parallel, transfectants were stimulated on Fc-ICAM-1-coated coverslips with CXCL12 for 30 min. Cells were fixed, permeabilized, and stained with TRITC-phalloidin. Whole-cell extracts were analyzed by Western blotting for the expression of SLP76, Vav1, and β-actin. One individual experiment of three is shown. D. Chemotaxis of human T cells transfected as described in A was addressed using a Transwell assay, as described in Materials and Methods. After 2 h, the number of migrated cells into the lower chamber was counted. Suppression of SLP76 expression was assessed by flow cytometry, and the percentage of SLP76-expressing cells was calculated. Data represent the mean and SE of three independently performed experiments.
response to CXCL12 stimulation (Fig. 7B). These experiments indicate that the LAT/Gads/SLP76 signaling platform is not assembled in response to CXCL12 in T cells.

Above we have shown that the ADAP/SKAP55/RIAM module inducibly interacts with SLP76 upon TCR-mediated T cell activation, and that SLP76 is mandatory for membrane targeting of the ADAP/SKAP55/RIAM module and for activation of Rap1 (Fig. 4). In marked contrast, CXCL12 stimulation of T cells did not lead to an inducible interaction between the LAT/Gads/SLP76 complex and the ADAP/SKAP55/RIAM module (Fig. 7B). However, the ADAP/SKAP55/RIAM module (and also Rap1) was properly recruited to the plasma membrane in response to CXCR4 stimulation in both SLP76-proficient and -deficient T cells (Fig. 7, C and D). Moreover, SLP76low Jurkat T cells displayed no obvious defect in CXCL12-mediated activation of Rap1 (Fig. 7E). These data suggest that SLP76 is not required for CXCR4-mediated Rap1 activation and plasma membrane targeting of ADAP, SKAP55, RIAM, and Rap1.

To exclude the possibility that ADAP and SKAP55 themselves are dispensable for CXCR4-regulated adhesion and migration, we down-regulated ADAP expression in human T cells by siRNA and subsequently assessed both the adhesiveness and migratory capacity of these cells in response to CXCL12. Fig. 7F shows that loss of ADAP strongly attenuated CXCR4-induced adhesion and chemotaxis in human T cells. Similar data were obtained when we assessed CXCR4-induced migration of ADAPlow Jurkat T cells or of T cells obtained from ADAP-deficient mice (data not shown). In summary, our data show that in contrast to TCR stimulation, SLP76 is not involved in membrane recruitment of the ADAP/SKAP55/RIAM module, the activation of Rac or Rap1, and the induction of adhesion or migration in response to CXCR4 stimulation.

Discussion
Together with the transmembrane adapter protein LAT, the cytosolic adapter protein SLP76 facilitates the formation of a signaling platform (LAT/Gads/SLP76) following TCR stimulation that leads to the activation of multiple intracellular signaling pathways (49). Our previous data suggested that the signaling pathways controlled by the Gads/SLP76 complex also include those regulating adhesion processes (22). In this study, we confirm this hypothesis by showing that suppression of SLP76 expression by RNAi in human T cells or the
Jurkat T cell line abrogates TCR-mediated conjugate formation, adhesion to ICAM-1, and affinity/avidity maturation of LFA-1. Consistent with previously published data (50, 51), we also found that loss of SLP76 attenuates adhesion to fibronectin (the ligand of β1 integrins; VLA-4) in response to TCR stimulation (J. Horn and S. Kliche, unpublished data). Thus, SLP76 regulates TCR-induced inside-out signaling, leading to the activation of β1 and β2 integrins.

Reorganization of the actin cytoskeleton is critically involved in TCR-mediated integrin activation (33, 34). Moreover, it has previously been demonstrated that overexpression of SLP76 enhances F-actin formation in response to TCR stimulation (52). In addition to these overexpression data, we show in this study that loss of SLP76 attenuates TCR-mediated polymerization of F-actin. Together these findings suggest that SLP76 is one of the (if not the) central molecule that links the TCR to remodeling of the actin cytoskeleton.

The defect in actin dynamics most likely results from an abrogated activation of the small GTPase Rac. Activation of Rac depends on membrane recruitment and activation of the GEF Vav1 and the Tec kinase Itk (20, 32). Both Vav1 and Itk associate with SLP76 upon TCR stimulation, and both molecules are required for F-actin formation at the IS, activation of Rac, clustering of LFA-1, adhesion to ICAM-1, and conjugate formation (20, 32). In this scenario, the role of Vav1 is to generate sufficient pools of activated Rac to promote F-actin polymerization through activation of proteins of the Wiskott-Aldrich syndrome protein/Wiskott-Aldrich syndrome-Verprolin-homologues protein family (33, 34). Our data showing attenuated actin reorganization in SLP76low cells are in line with a model in which loss of SLP76 blocks activation of Rac (and consequently signaling events downstream of Rac) by impairing the functions of Vav1 and Itk.

We are, however, aware that another group recently reported that TCR-mediated actin remodeling is not attenuated in the SLP76-deficient Jurkat T cell line J14 (53). In addition, no defect in Rac activation upon TCR stimulation was observed in this Jurkat variant (54). The reasons for these discrepant findings in SLP76-deficient Jurkat T cells vs SLP76low human T cells are unclear at present. A possible explanation may come from compensatory mechanisms that developed in line J14 to escape the selection pressure that cannot develop in short-term manipulated T cells.

Besides the reorganization of the actin cytoskeleton, one critical regulator of TCR-mediated conjugate formation and inside-out signaling is the small GTPase Rac. Several studies have reported that Rap1 activation critically depends on the expression of PLCγ1 (13, 26, 55). PLCγ1 signaling to Rap1 occurs via the nucleotide exchange factor calcium and diacylglycerol-regulated guanine nucleotide exchange factor I that is activated by the second messengers calcium and diacylglycerol (which both are generated following phosphatidylinositol 4,5-bisphosphate hydrolysis by PLCγ1). Thus, a defect in PLCγ1 activation in SLP76-suppressed T cells (see Fig. 1C and supplemental Fig. 2, C and D) and consequently a failure to activate CalDAG-GEF1 might be largely responsible for the attenuated activation of Rap1 after TCR triggering in the absence of SLP76. However, additional mechanisms controlled by SLP76 may also contribute to the block in Rap1 activation. For example, SLP76 is mandatory for activation and membrane targeting of protein kinase Cθ, which has recently been identified to control TCR-induced Rap1 activity by phosphorylation of Rap1-GEF2 (56, 57). Hence, loss of SLP76 might affect several signaling pathways that are critical for TCR-induced activation of Rap1.

We had previously shown that the ADAP/SKAP55/RIAM complex is required for plasma membrane targeting of Rap1. Moreover, we had demonstrated that this event is crucial for inside-out signaling and for the activation of integrins in response to TCR stimulation. However, loss of ADAP and/or SKAP55 selectively abrogated plasma membrane recruitment of Rap1, although TCR-mediated activation of Rap1 was not affected (17, 21). In contrast, the data presented in this study show that SLP76 is mandatory for both membrane targeting and activation of Rap1 after TCR triggering. The former event most likely involves the ADAP/SKAP55/RIAM module, whereas the activation of Rap1 might be regulated through the PLCγ1- or the protein kinase Cθ-signaling pathways that were discussed above. Hence, our data support a model in which plasma membrane targeting and activation of Rap1 are two interrelated, but distinctly controlled signaling events that are organized at the level of SLP76.

The activation of Rap1 and Rac as well as changes in F-actin dynamics are also mandatory for affinity modulation of LFA-1, T cell adhesion, and migration in response to CXCR4 stimulation (6, 58). Given the central role of SLP76 in TCR-mediated signaling, it was totally unexpected for us to find that loss of SLP76 did not affect these events after CXCR4 stimulation and that the SLP76/Gads complex was not recruited to LAT in response to CXCR4 stimulation. The latter observation is probably due to the fact that the tyrosine residue within LAT that is responsible for recruiting the Gads/SLP76 complex (Y171) is not phosphorylated in response to CXCL12 triggering.

Our data also showed that SLP76 is not involved in the recruitment (and consequently the activation) of downstream effector molecules (e.g., Vav1) that are important for mediating adhesion and migration processes of T cells in response to CXCL12 triggering (58). Indeed, whereas Vav1-deficient T cells failed to polarize in response to CXCL12 stimulation, SLP76low T cells showed no defect in this process. Moreover, CXCL12-induced lateral migration velocity and chemotaxis were unaffected in SLP76low T cells. Hence, it appears as if SLP76 is dispensable for both adhesive and migratory processes in response to CXCL12 stimulation.

In line with the functional data, we found that membrane recruitment of the ADAP/SKAP55/RIAM module that is critical for CXCR4-mediated adhesion and migration of T cells was not affected by loss of SLP76. Although this finding strongly supports our above discussed hypothesis that membrane targeting of Rap1 and activation of this GTPase are distinct processes that are individually regulated by different signaling receptors, it opens the question as to which signaling molecule provides the molecular link between CXCR4 and the ADAP/SKAP55/RIAM module.

In this regard, it has recently been shown that the cytosolic adapter protein Shc is phosphorylated upon CXCR4 stimulation and assembles into a complex that includes Lck, ZAP70, Vav1, and LAT (59). Moreover, Shc-deficient Jurkat T cells show an attenuated migratory capacity and impaired F-actin dynamics upon CXCR4 stimulation, and mutation of critical tyrosine residues of Shc (Y238/240 or Y317) results in defective phosphorylation of Vav1 and Itk (59). Thus, Shc might be an attractive candidate to connect CXCR4 with the Vav1-Itk-dependent pathway of F-actin polymerization and T cell migration (59). It will be important to assess the role of Shc in CXCR4-induced inside-out signaling, adhesion, and Rap1 activation in future studies. Similarly, it needs to be analyzed whether, and if so how, LAT is involved in this process. Experiments are currently set up in our laboratory to assess these points. Nevertheless, our data collectively and surprisingly indicate that, in contrast to TCR signaling, SLP76 is not a key component connecting the chemokine receptor CXCR4 with the activation of integrins and the induction of T cell adhesion and migration.
Disclosures

The authors have no financial conflict of interest.

References


