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The Src Homology 2 Domain-Containing Leukocyte Protein of 76-kDa Adaptor Links Integrin Ligation with p44/42 MAPK Phosphorylation and Podosome Distribution in Murine Dendritic Cells

Nancy A. Luckashenak,* Rebecca L. Ryszkiewicz,* Kimberley D. Ramsey,† and James L. Clements2*

The Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) is an important molecular intermediate in multiple signaling pathways governing immune cell function. In this study, we report that SLP-76 is expressed in CD11c−B220− dendritic cells (DCs) isolated from murine thymus or spleen, and that SLP-76 is rapidly phosphorylated on tyrosine residues upon plating of bone marrow-derived DCs (BMDCs) on integrin agonists. SLP-76 is not required for the in vitro or in vivo generation of DCs, but SLP-76-deficient BMDCs adhere poorly to fibronectin, suggesting impaired integrin function. Consistent with impaired adhesion, cutaneous SLP-76-deficient DCs leave ear tissue at an elevated frequency compared with wild-type DCs. In addition, the pattern and distribution of actin-based podosome formation are visibly altered in BMDCs lacking SLP-76 following integrin engagement. SLP-76-deficient BMDCs manifest multiple signaling defects following integrin ligation, including reduced global tyrosine phosphorylation and markedly impaired phosphorylation of p44/42 MAPK (ERK1/2). These data implicate SLP-76 as an important molecular intermediate in the signaling pathways regulating multiple integrin-dependent DC functions, and add to the growing body of evidence that hemopoietic cells may use unique molecular intermediates and mechanisms for regulating integrin signaling. The Journal of Immunology, 2006, 177: 5177–5185.

Dendritic cells (DCs) are central to establishing and maintaining peripheral tolerance to self as well as directing immune responses to foreign pathogens in an inflammatory context. DC function is critically dependent on anatomical distribution and migratory capacity, which in turn are regulated in large part through the concerted action of chemokine receptors and integrins. Interestingly, hemopoietic and nonhemopoietic cells differ in their cytoskeletal response to integrin ligation. This is demonstrated perhaps most dramatically by the absence of stress fibers and focal adhesions in hemopoietic cells, and may reflect the highly mobile phenotype characteristic of hemopoietic cells in general. This raises the possibility that integrin function in the immune system may be regulated by a unique set of signaling molecules restricted in expression to hemopoietic cells.

The Src homology 2 domain-containing leukocyte protein of 76-kDa (SLP-76) adaptor protein is expressed in multiple hemopoietic cell types, and functions as a positive regulator in numerous signaling pathways downstream of surface receptor ligation. The current paradigm for SLP-76-dependent signaling applies primarily to receptors coupled to polypeptide chains containing one or more ITAMs (3). For example, ligation of the TCR leads to rapid activation of several Src family protein tyrosine kinases (PTKs), which phosphorylate tyrosine residues within multiple ITAMs in the associated CD3 (γ, δ, and ε-) and ζ-chains (4). This results in the recruitment and activation of Zap-70 (a Syk family PTK), which then phosphorylates SLP-76 and the linker for activation of T cells (LAT) (5, 6). The inducible phosphorylation of SLP-76 and LAT following TCR ligation drives the formation of multimolecular signaling complexes that are critical for subsequent downstream signaling events, including phospholipase Cγ1 (PLCγ1) activation, MAPK phosphorylation (ERK1/2), cytokine gene transcription (e.g., IL-2), and dynamic changes in the actin and microtubule cytoskeletons (6–13).

In addition to ITAM-coupled receptor systems, SLP-76 also functions downstream of integrin ligation in platelets and neutrophils (14, 15). However, the mechanisms by which SLP-76 functions to promote integrin signaling are less well defined, and may involve molecular intermediates and targets that are both common to and distinct from those described for ITAM-bearing receptor systems such as the TCR and the collagen receptor (GPVI). In this study, we provide the first evidence that SLP-76 is expressed in murine CD11c−B220− DCs isolated from thymus or spleen and functions to regulate integrin signaling in DCs. SLP-76-deficient DCs generated from bone marrow exhibit impaired adhesion and altered patterns of actin assembly and podosome distribution following integrin ligation, implicating SLP-76 as a central regulator of the actin cytoskeleton following integrin ligation. Additionally,
several integrin-triggered signaling events are disrupted in the absence of SLP-76, including inducible phosphorylation of ERK1/2 MAPKs. These data implicate SLP-76 as an important molecular intermediate in multiple signaling pathways triggered by integrin ligation in murine DCs, and highlight SLP-76 as one focal point for defining the molecular mechanisms governing integrin signaling and function in hemopoietic cells.

Materials and Methods

Mice

C57BL/6 and Rag-2−/− mice on a C57BL/6 background were obtained from The Jackson Laboratory. SLP-76-deficient mice (C57BL/6 × 129, H-2b) were generated, as described (16). Animals were maintained under specific pathogen-free conditions in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility in accordance with the policies and guidelines set forth by the Roswell Park Cancer Institute Institutional Animal Care and Use Committee.

Isolation of thymic and splenic DCs

DCs were enriched from thymus or spleen using previously described protocols with minor modifications (17, 18). In brief, the spleen or thymus of 8- to 12-week-old mice was harvested, minced thoroughly, and digested in RPMI 1640 (5% FCS) containing collagenase D (1–2 μg/ml; Roche Pharmaceuticals) for 30 min at 37°C with gentle agitation. Digested tissues were passed through a 70-μm nylon cell strainer, washed once, and resuspended in PBS/0.2% BSA and 2 mM EDTA, and then layered onto a 70% Percoll solution (60% (w/v) iodixanol; Nyegaard Diagnostics) diluted in HBSS containing 0.2% BSA and 2 mM EDTA. The low-density cell fraction was isolated from thymus or spleen, as described. The low-density cell fraction was present at the interface between the 12% Optiprep solution and the HBSS was harvested following centrifugation at ~700 × g for 20 min at 25°C and resuspended in RPMI 1640 supplemented with 5% FCS.

Analysis of SLP-76 expression by flow cytometry

The low-density cell fraction was isolated from thymus or spleen, as described, and stained with fluorochrome-conjugated Abs (BD Biosciences/Bd Pharmingen) specific for CD11c (clone HL3), B220 (CD45R, clone RA3-6B2), or CD8 (clone 53-2.1; all from BD Biosciences/BD Pharmingen) and either a FITC- or PE-conjugated Ab specific for SLP-76 (eBioscience). Analysis was performed on a FACScan flow cytometer, and collected data were analyzed using WinMDI 2.8 software.

RT-PCR

Low-density cell fractions were collected from collagenase-digested thymus or spleen suspensions, as described, and cells were stained with a combination of fluorochrome-conjugated Abs specific for CD11c, B220, CD19 (clone 1D3), and Thy-1.2 (clone 53-6.7). Stained cells were fixed with 2% paraformaldehyde, permeabilized (IC-Perm buffer; BioSource International), and incubated with a FITC-conjugated isotype (IgG2A) control Ab or a FITC-conjugated Ab specific for SLP-76 (eBioscience). Analysis was performed on a FACSscan flow cytometer, and collected data were analyzed using WinMDI 2.8 software.

Semiquantitative PCR was performed (25, 30, or 35 cycles) with primers and primer combinations specific for murine SLP-76 (5'-ACTCGTTCTCAGGAGGAGC-3', reverse 5'-CTTGCCGCTCAGGAGGAGC-3', reverse 5'-CATAGCAGGCAGGCATGCGGT-3'; B cell linker protein (BLNK) (SLP-65)); forward 5'-CATGCTGAGAATGTGCAACG-3', reverse 5'-GCTGATGACGTTCATGACGAG-3', or actin: forward 5'-ATGGAATCTCTTTGGCAT-3', reverse 5'-ACTTTGGCTCAGGAGGACC-3'.

Generation of bone marrow-derived DCs (BMDCs)

DCs were generated from bone marrow harvested from wild-type or SLP-76-deficient mice using standard protocols with minor modifications. Briefly, femurs and tibias were harvested from 8- to 12-wk-old mice was harvested, minced thoroughly, and digested in RPMI 1640 supplemented with 10% FCS and murine GM-CSF (20 ng/ml, isolated as a cell culture supernatant) for 7–8 days. Approximately 75% of the medium was removed on days 2 and 4 and replaced with fresh medium containing GM-CSF. BMDCs harvested at day 7 were cultured for an additional 20 h in the presence of LPS at 1 μg/ml (Sigma-Aldrich) to promote maturation. Cell phenotype was routinely monitored by flow cytometry using a panel of Abs that reflect murine DC lineage and maturation, including CD11c, CD80 (16-10A1), CD86 (GL1), CD40 (3/23), H-2K\(^{\text{a}}\) (AF6-88.5), CD11b (M1/70), and I-A/E (M5/114/15.2), purchased from BD Biosciences/Bd Pharmingen or Biologend. For analysis of integrin β-1 chain expression, Abs to CD29 (Ha2/5; BD Biosciences/Bd Pharmingen) and CD18 (C71/16; Caltag Laboratories) were used.

Integrin stimulation, immunoblotting, and immunoprecipitation

LPS-matured BMDCs were harvested and washed extensively to remove LPS from the culture medium. Washed cells were rested in medium without out FCS at 37°C for up to 20 h and left in suspension or plated in 10-cm petri dishes precoated with poly-L-lysine (100 μg/ml; Sigma-Aldrich), fibronectin (10 μg/ml; Sigma-Aldrich), RGDs peptide (15 μg/ml; Bachem), or an Ab specific for CD18 (clone 71/16, 5 μg/ml; Caltag Laboratories). Where indicated, the Syk inhibitor picenatel (Sigma-Aldrich) was added at a final concentration of 50 nM. Adherent and nonadherent cells were collected and lysed at specific time points in 500 μl of radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Lysates were incubated with 0.5 μg of mouse IgG2a (Biologend MOPC-173) for 30 min, followed by the addition of protein G-Sepharose beads (Zymed Laboratories) for 2 h. Nonspecific complexes were cleared from the lysate by centrifugation and removal of the Sepharose beads. Abs specific for SLP-76 (2.5 μg; Ab Solutions) or Yav (1 μg, C14:sc132; Santa Cruz Biotechnology) were added to the precleared lysates and incubated overnight, followed by addition of protein G-Sepharose beads and an additional 2-h incubation. Precleared lysates and immunoprecipitates were then incubated at 4°C with constant rotation. Western blots of immunoprecipitated proteins or whole cell lysates were performed using Abs specific for SLP-76 (polyclonal rabbit antisera; BioSource International), Yav (C14:sc132; Santa Cruz Biotechnology), phosphotyrosine (4G10; Upstate Biotechnology), phospho-p44/p42 MAPK (E10, p44/p42 MAPK, phospho-PLC-ζ2, PLCγ2 (Cell Signaling Technology), phospho-focal adhesion kinase (FAK; Tyr397; Upstate Biotechnology), or FAK (H-1; Santa Cruz Biotechnology). Appropriate HR-conjugated secondary Abs (Jackson ImmunoResearch Laboratory) were used in conjunction with a chemiluminescent substrate (SuperSignal West Pico; Pierce) for protein visualization.

Adhesion assays

Adhesion assays were performed in flat-bottom 96-well, nonstick culture-treated plates precoated with bovine fibronectin (20 μg/ml) or 1% BSA. Coated wells were blocked with 1% BSA at 37°C for additional 1 h. LPS-treated BMDCs were harvested and rested in medium without FCS at 37°C for 1 h before plating. A total of 10⁵ cells was added to the wells and allowed to adhere for 1.5 h at 37°C. Nonadherent cells were removed by washing wells three to four times with medium without FCS. The adherent cells were fixed in 2% paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich) for 25 min at 25°C. Excess stain was removed by washing wells three to four times with medium without FCS. The adherent cells were fixed in 2% paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich) for 25 min at 37°C. Excess stain was removed by submersion in water, and wells were allowed to air dry for 10 min. Stained cells were photographed after drying using an inverted microscope equipped with a Nikon Eclipse TE300 camera with a ×10 objective. The cells were lysed in 0.5% Triton X-100 overnight at 25°C, and the OD was determined at 595 nm using a microtiter-plate reader (Dyntech Laboratories).

Ear explant cultures and in situ staining of Langerhans cells

Migration was examined using an adaptation of an assay originally developed by Larsen et al. (19). Ears were removed from mice, weighed, and split into dorsal and ventral halves using forceps. The dorsal half was cultured in medium containing 100 ng/ml CCL21 (R&D Systems) for 24 h at 37°C. Migrated cells were collected, and the process was repeated for another 24 h using fresh medium and chemokine. All migrated cells from one individual dorsal ear preparation were pooled and counted. To visualize Langerhans cells, the dorsal and ventral ear halves were isolated and incubated at 37°C (dermal side down) for 20 min in 0.5 M ammonium thioscianate (Sigma-Aldrich). Epidermal sheets were carefully isolated using forceps and rinsed in PBS. The sheets were then fixed in acetone for 5 min and stained with a PE-conjugated Ab against murine I-A/E (M5/114/15.2; Biologend). Samples were photographed using an inverted fluorescent microscope equipped with a Nikon Eclipse TE300 camera and a ×10 objective.

Transwell migration assay

To assess BMDC chemotaxis, 5 × 10⁵ cells (LPS treated) were added to the upper chamber of a 6-well plate containing 5-μm-pore polycarbonate filters (Transwell; Costar). Medium alone (without FCS) or medium plus the chemokine CCL21 (10 nM final) was added to the bottom wells, and the plate was incubated for 1.5 h at 37°C. Cells that migrated into the...
bottom well were collected, stained for CD11c, and enumerated by flow cytometry using PKH26 reference microbeads (Sigma-Aldrich).

**Analysis of actin filament assembly and podosome formation**

Glass coverslips were coated with RGDS (15 μg/ml) or fibronectin (30 μg/ml) overnight at 4°C. The following day, LPS-treated DCs were harvested and rested in medium without FCS at 37°C for 1 h. A total of 8 × 10⁵ DCs was plated onto the prewarmed, coated coverslips and allowed to adhere for 45 min at 37°C. Coverslips were rinsed with PBS, and adherent cells were fixed in 4% paraformaldehyde for 20 min (all staining procedures took place at 25°C). Cells were permeabilized in 0.5% Triton X-100 for 5 s and then blocked in 1% BSA for 30 min. Cells were stained with Alexa Fluor 488 phalloidin (Molecular Probes) in blocking buffer for 45 min (1 μl/coverglass). Some samples were costained with a vinculin-specific Ab (hVIN-1; Sigma-Aldrich), followed with a rhodamine-conjugated goat anti-mouse secondary Ab for visualization. Coverslips were mounted, and confocal microscopy was performed using a Leica SP2 laser-scanning confocal microscope. Images were acquired at ×63.

**Statistical analysis**

Data were analyzed for averages and SDs using Microsoft Excel. Statistical significance was determined using a paired two-tailed Student’s t test. Results were considered to be statistically significant when p < 0.05.

**Results**

SLP-76 is expressed in primary CD11c⁺ B220⁻ DCs isolated from thymus or spleen

Several subsets of DCs with unique properties and functions have been described in mouse and human (reviewed in Refs. 20 and 21). SLP-76 is expressed in multiple myeloid and lymphoid lineage cell types, including macrophages, mast cells, and T cells (22–24). However, expression and function of SLP-76 in DCs have not been described. To assess SLP-76 protein expression in freshly isolated murine DCs, we first enriched the more buoyant fraction of cells from murine spleen or thymus and then analyzed SLP-76 expression in several distinct DC subsets by flow cytometry (Fig. 1). SLP-76 expression was readily detected in the CD11c⁺ B220⁻ DC subset enriched from both spleen and thymus (see population I; Fig. 1, A and B). The level of SLP-76 expression in the CD11c⁺ B220⁻ DC subset was comparable to that observed in resting peripheral T cells (data not shown). In contrast, SLP-76 is expressed at relatively low levels in the CD11c⁺ B220⁺ DC subset, particularly in the thymus (population II; Fig. 1, A and B). The CD11c⁺ B220⁻ subset (population III) consists predominantly of B cells, and provides a useful internal reference, as mature B cells express very low levels of SLP-76 (25). No obvious differences in SLP-76 expression were found when thymic or splenic DCs (CD11c⁺) were subgated further based on CD4 or CD8 coreceptor expression (data not shown). These data indicate that SLP-76 is expressed in the CD11c⁺ B220⁻ DC subset in both thymus and spleen, with comparatively little expression in the plasmacytoid-like DC population (CD11c⁻ B220⁻).

We next examined sort-purified thymic and splenic DC subsets for the presence of SLP-76-specific mRNA transcripts using semi-quantitative PCR (Fig. 1, C and D). Consistent with the flow cytometric analysis of SLP-76 expression, SLP-76-specific PCR products were more readily amplified from the CD11c⁺ B220⁻ DC subset isolated from spleen or thymus when compared with the CD11c⁺ B220⁺ subset (Fig. 1, C and D). Still, mRNA encoding SLP-76 was detectable in the CD11c⁺ B220⁺ subsets, albeit at lower levels than that detected in the CD11c⁺ B220⁻ subset. Conversely, mRNA encoding the SLP-76 homologue BLNK/SLP-65 was reproducibly detected in thymic and splenic CD11c⁺ B220⁺ DCs, but was not appreciably amplified from RNA isolated from the CD11c⁺ B220⁻ population. A low level of BLNK/SLP-65 mRNA was reproducibly detected in thymic CD11c⁺ B220⁻ DCs. This was most likely not due to B cell contamination, as no CD19-specific transcripts were detectable in any of our sorted DC populations (data not shown).

Contamination of our sorted DC populations with T cells was also negligible, as primers specific for cDNA encoding the LAT adaptor protein failed to amplify any detectable product in CD11c⁺ B220⁻ or CD11c⁺ B220⁺ DC populations (data not shown). Collectively, these data provide the first evidence that SLP-76 is expressed in freshly isolated DCs, and indicate further that the structurally homologous SLP-76 and BLNK/SLP-65 adaptor proteins are differentially expressed in murine DC subsets.

**SLP-76 is not strictly required for the in vivo development of splenic DCs**

We next determined whether SLP-76 is required for the development and/or differentiation of several in vivo DC subsets. Although CD11c⁺ DCs are clearly present in the thymus of SLP-76-deficient animals (Fig. 2A), these cells display an altered phenotype that is more comparable to CD11c⁻ DCs expressing very low levels of SLP-76 (25). No obvious differences in SLP-76 expression were found when thymic or splenic DCs (CD11c⁺) were subgated further based on CD4 or CD8 coreceptor expression (data not shown). These data indicate that SLP-76 is expressed in the CD11c⁺ B220⁻ DC subset in both thymus and spleen, with comparatively little expression in the plasmacytoid-like DC population (CD11c⁻ B220⁻). Though CD11c⁻ expression, with a high percentage of the CD11c⁺ cells expressing abnormally low levels of CD8α. A reduced level of CD8α has been reported previously for RAG-2-deficient thymic DCs (26). Indeed, when comparing directly thymic DCs isolated from RAG-2-deficient or SLP-76⁻⁻ mice, the phenotypes were found to be remarkably similar (Fig. 2A). This suggests that the phenotype of SLP-76-deficient thymic DCs is not cell intrinsic, but more likely a consequence of an altered thymic microenvironment common to the SLP-76 and RAG-2 strains (e.g., lack of mature thymocytes). This notion is supported further by the observation that CD11c⁺ DCs isolated from mice deficient for LAT display a phenotype that is also virtually identical with that observed in SLP-76⁻⁻ or RAG-2-deficient mice (data not shown). Despite the abnormal phenotype observed in the thymus, splenic CD11c⁺ DCs isolated from SLP-76-deficient animals demonstrate a phenotype that is more comparable to CD11c⁻ DCs

**FIGURE 1.** SLP-76 is expressed in thymic and splenic CD11c⁺ B220⁻ DCs. A and B, Low-density cells were isolated from spleen (A) or thymus (B) preparations, surface stained for CD11c in combination with B220 (CD45R), and analyzed for intracellular SLP-76 expression by flow cytometry. The mean fluorescence intensity (MFI) with background fluorescence subtracted (mouse IgG2A) is provided for each population. C and D, The indicated DC populations were sort purified (>98%) from spleen (C) or thymus (D) and analyzed for SLP-76, BLNK (SLP-65), or actin-specific transcripts using RT-PCR (25, 30, or 35 cycles).
SLP-76 is not strictly required for the generation of in vivo thymic or splenic DCs. A, The low-density cell fraction was enriched from cell suspensions prepared from thymus harvested from control (SLP-76+/+), SLP-76-deficient (SLP-76−/−), or RAG-2-deficient mice and stained for CD11c and CD86 expression. B, Surface phenotype of DCs enriched from SLP-76−/− or SLP-76+/+ splenic cell preparations. The percentage of cells contained within a given quadrant is indicated.

Harvested from control mice (C57BL/6), including roughly equivalent surface expression of CD8α, MHC class II, and B220 (Fig. 2B, data not shown). Thus, SLP-76 does not appear to be strictly required for the in vivo development of the CD11c+ DC subsets analyzed in this study. However, the global loss of SLP-76 in the hematopoietic compartment clearly results in an altered DC phenotype, particularly in the thymus, that is most likely a consequence of DC extrinsic factors.

SLP-76 is expressed in BMDCs and is rapidly phosphorylated following integrin engagement

Given the relative paucity and heterogeneous nature of in vivo DC subsets, and potential extrinsic effects of an SLP-76-deficient microenvironment on DC development and function, we wished to determine whether BMDCs might serve as a useful model to begin to define SLP-76-dependent signaling pathways in DCs. Cultures generated from murine bone marrow harvested from wild-type mice routinely consisted of >90% CD11c+ cells (Fig. 3A, left panel). These cells expressed elevated levels of MHC class II and costimulatory molecules (e.g., B7-2) following LPS exposure, consistent with a mature DC phenotype (Fig. 3A, right panel, and data not shown). As expected, SLP-76 expression was detected (by flow cytometry) in wild-type BMDCs at a level comparable to that revealed in freshly isolated thymic or splenic DCs (data not shown). No obvious difference in the level of SLP-76 expression was noted following LPS exposure or between immature (B7-2low) and mature (B7-2high) CD11c+ BMDCs.

Given the recent reports describing SLP-76 phosphorylation and function following integrin engagement in platelets and neutrophils (15, 27), we next determined whether SLP-76 is phosphorylated in BMDCs in response to integrin ligation. To this end, we next determined whether SLP-76 is phosphorylated in response to integrin ligation. A, BMDCs were prepared from wild-type mice and stained for CD11c and CD86 (B7-2) at the conclusion of a 7-day culture (PRE-LPS) or following an additional 20-h incubation in the presence of LPS (POST-LPS). Cells were analyzed using flow cytometry, and the percentage of cells present in each quadrant is indicated. B, Wild-type LPS-treated BMDCs were rested for 20 h and then left in suspension (S) for 5 min or stimulated with plate-bound RGDS for the indicated times. SLP-76 was immunoprecipitated from cell lysates and assessed for tyrosine phosphorylation by Western blotting (upper panel). The membrane was stripped and rebalotted with a SLP-76-specific Ab (lower panel). C, Same as above, except cells were stimulated with plate-bound poly(L-lysine) (PLL) or RGDS for 10 min before lysis and SLP-76 immunoprecipitation. Where indicated, cells were preincubated with piceatannol before stimulation (+PIC). In addition to blotting for phosphotyrosine (P-Tyr, upper panel) and SLP-76 (middle panel), membranes were also stripped and rebalotted with an Ab specific for Vav (bottom panel).

SLP-76-deficient DCs adhere poorly to fibronectin and exit ear tissue at an elevated frequency in the presence of chemokine

The observation that SLP-76 is phosphorylated and associates with Vav following integrin ligation, A, BMDcs were prepared from wild-type mice and stained for CD11c and CD86 (B7-2) at the conclusion of a 7-day culture (pre-LPS) or following an additional 20-h incubation in the presence of LPS (post-LPS). Cells were analyzed using flow cytometry, and the percentage of cells present in each quadrant is indicated. B, Wild-type LPS-treated BMDCs were rested for 20 h and then left in suspension (S) for 5 min or stimulated with plate-bound RGDS for the indicated times. SLP-76 was immunoprecipitated from cell lysates and assessed for tyrosine phosphorylation by Western blotting (upper panel). The membrane was stripped and rebalotted with a SLP-76-specific Ab (lower panel). C, Same as above, except cells were stimulated with plate-bound poly(L-lysine) (PLL) or RGDS for 10 min before lysis and SLP-76 immunoprecipitation. Where indicated, cells were preincubated with piceatannol before stimulation (+PIC). In addition to blotting for phosphotyrosine (P-Tyr, upper panel) and SLP-76 (middle panel), membranes were also stripped and rebalotted with an Ab specific for Vav (bottom panel).

SLP-76-deficient DCs adhere poorly to fibronectin and exit ear tissue at an elevated frequency in the presence of chemokine

The observation that SLP-76 is phosphorylated and associates with Vav in BMDCs following integrin engagement suggested the possibility that SLP-76 may be an important intermediate in one or more signaling pathways governing integrin function. In support of this idea, SLP-76-deficient BMDCs adhere poorly to a fibronectin-coated culture surface when compared with wild-type BMDCs (Fig. 4, A and B). Similar results were obtained using freshly isolated CD11c+ splenic DCs obtained from SLP-76 wild-type and SLP-76-deficient mice (data not shown). The poor adhesion manifested by the SLP-76-deficient BMDCs is most likely not due to alterations in integrin surface expression, as the levels of the β1 integrin chain (CD29), the β2 integrin chain (CD18), and several β2-containing integrins (CD11c and CD11b) are comparable between wild-type and SLP-76-deficient BMDCs (data not shown). Importantly, no obvious difference in surface phenotype (pre- or post-LPS), cell yield, or viability was observed when comparing...
SLP-76-deficient BMDCs display an altered pattern of podosome distribution following integrin ligation. To visualize F-actin, LPS-treated BMDCs generated from wild-type (A and C) or SLP-76-deficient (B and D) mice were rested for 4–6 h and plated on RGDS-coated glass coverslips for 45–60 min and visualized F-actin using confocal microscopy. Following a 45-min incubation, the majority of wild-type BMDCs plated on RGDS-coated coverslips maintained a nonpolarized phenotype, and many (~30–40%) of these displayed a striking array of small, F-actin-based clusters or foci distributed evenly throughout the plane of contact with the coverslip (Fig. 5, A and C) that colocalized with the actin-bundling protein vinculin (Fig. 5C, inset). This distribution and pattern of F-actin and vinculin assembly are consistent with that described for podosomes: actin-rich adhesive structures observed predominantly in motile cells of the myeloid lineage (macrophages, osteoclasts, DCs), virally transformed fibroblasts, and malignant B cells (33). In marked contrast to the dispersed pattern of podosomes observed in wild-type BMDCs, the majority of SLP-76-deficient BMDCs that assemble podosomes arrange these structures in more concentrated ring-like configurations within the cell (see filled arrowheads; Fig. 5B). The F-actin-based clusters formed by the SLP-76-deficient DCs also colocalize with vinculin (Fig. 5D, inset), and are very similar to podosome-containing structures termed rosettes observed in maturing osteoclasts and Rous sarcoma virus-transformed fibroblasts (34, 35). These data suggest that SLP-76 is dispensable for initial podosome formation, but is required for the regulated distribution of podosomes.
throughout the contact surface. The rosette-like pattern of podosomes observed in SLP-76-deficient BMDCs may also be related to the reduced adhesive capacity of these cells, and could therefore be an indirect consequence of SLP-76 deficiency.

**Globally reduced tyrosine phosphorylation and defective ERK1/2 MAPK phosphorylation in SLP-76-deficient BMDCs following integrin engagement**

The impaired adhesion and irregular distribution of podosomes observed in SLP-76-deficient BMDCs suggested that SLP-76 functions to couple integrin ligation with more distal signaling events in DCs. To begin to identify specific SLP-76-dependent aspects of integrin signaling in DCs, we first analyzed global induction of tyrosine phosphorylation in LPS-treated BMDCs generated from control or SLP-76−/− mice plated for varying times in RGDS-coated tissue culture wells. As expected, integrin ligation results in the enhanced phosphorylation of multiple proteins in BMDCs generated from wild-type mice (Fig. 6A). In the absence of SLP-76, there is a visible induction of protein tyrosine phosphorylation following integrin ligation, but the levels of phosphorylation do not approach that observed in wild-type BMDCs (Fig. 6A). These data are consistent with the observation that the total cellular levels of phosphotyrosine are also reduced in SLP-76-deficient platelets plated on fibrinogen-coated coverslips (27), and suggest that SLP-76 is required for the following: 1) optimal activation of specific PTKs; and/or 2) the localization of activated PTKs and their specific substrates following integrin engagement.

Generally reduced levels of tyrosine phosphorylation discussed above, the basal and integrin-stimulated levels of PLCγ2 tyrosine phosphorylation were also diminished in SLP-76-deficient BMDCs (Fig. 6, B and C). Similar to PLCγ2, the steady state and integrin-stimulated levels of Vav1 phosphorylation were also reduced in the absence of SLP-76, although not to the degree observed for PLCγ2 (data not shown). These data are consistent with what has been reported previously in SLP-76-deficient neutrophils (15). Syk-deficient neutrophils also manifest defective Vav phosphorylation upon integrin engagement (36), suggesting that Syk and SLP-76 may cooperate in promoting optimal Vav (and possibly PLCγ2) phosphorylation downstream of integrin ligation in hemopoietic cells. Although somewhat variable, phosphorylation of FAK on the tyrosine at position 397 (Tyr397) remained largely intact in SLP-76-deficient BMDCs plated on poly(RGDS) (data not shown), indicating that SLP-76 is not strictly required for FAK phosphorylation in BMDCs plated on integrin agonists.

**SLP-76 is required for integrin-dependent phosphorylation of ERK1/2 MAPKs in murine BMDCs**

We next examined a potential role for SLP-76 in regulating MAPK activity following integrin ligation in BMDCs. Although a robust, but transient phosphorylation of the ERK1/2 MAPKs was readily detected in wild-type or SLP-76-deficient BMDCs stimulated with plate-bound RGDS or Abs specific for CD18, there was a marked reduction in the inducible phosphorylation of ERK1/2 in the absence of SLP-76 in both cases (Fig. 7). This result demonstrates that SLP-76 is required for coupling integrin ligation with more distal signaling events in DCs, and suggests further that the downstream effects of integrin-regulated ERK1/2 activation may also be compromised in the absence of SLP-76.

**Discussion**

Integrin signaling is remarkably complex, and involves multiple receptor:ligand combinations and molecular intermediates. In non-hemopoietic cells, Src family PTKs and FAK play central roles in propagating integrin signaling by phosphorylating numerous downstream substrates and, particularly in the case of FAK, nucleating the assembly of multimolecular signaling complexes at...
the site of focal adhesion/contact. Although similar signaling mechanisms involving Src family PTKs, FAK, and the FAK-homologue Pyk2 are most likely at work in cells of the immune system, the expression of hemopoietic-specific signaling intermediates such as SLP-76 may confer unique signaling properties upon integrins expressed on immune cells. This could account, at least in part, for the distinct adhesive structures and highly motile nature of immune cells. Indeed, integrins play central roles in multiple aspects of immune system function, including extravasation of immune cells from the bloodstream and the formation of stable and productive conjugates between T cells and APCs. It is also likely that integrins play additional roles in regulating immune cell growth and differentiation as well. Previous reports demonstrating a requisite role for SLP-76 in integrin signaling in platelets and neutrophils (15, 27), coupled with this work describing SLP-76 function in multiple integrin-triggered signaling pathways in DCs, support the idea that SLP-76 and SLP-65-associated proteins (e.g., Vav) provide integrins with distinct signaling mechanisms that are not generally available to nonhemopoietic cells. Still, the questions of how these proposed hemopoietic-specific pathways link integrin ligation with more distal outcomes (e.g., podosomes, motility) and integrate with other well-defined integrin-triggered pathways (e.g., FAK dependent) remains an open area of investigation.

Before this study, expression and/or function of SLP-76 in distinct DC subsets had not been addressed. Although expression of SLP-76 in the CD11c⁺ B220⁺ DC subset in thymus and spleen was not surprising; it is intriguing that SLP-76 and the structurally related adaptor BLNK/SLP-65 are differentially expressed in the B220⁻ and B220⁺ DC subsets. In T and B cells, SLP-76 and BLNK/SLP-65 clearly function in similar signaling pathways following TCR or BCR ligation, respectively. Although SLP-76 has emerged as an important intermediate in the signaling pathways downstream of integrin ligation, a comparable role for BLNK/SLP-65 in regulating integrin function has not been described. Even if BLNK/SLP-65 can support integrin signaling in a manner analogous to SLP-76, the observation that BLNK/SLP-65 is not appreciably expressed (at the mRNA level) in the CD11c⁺ B220⁻ DC subset indicates that there is most likely no molecular means to compensate for the loss of SLP-76 in this subset.

Given the observed interaction between Vav and SLP-76 in wild-type BMDCs stimulated on poly(RGDS) and the altered actin cytoskeleton distribution in BMDCs lacking SLP-76, it seems likely that one major function of SLP-76 downstream of integrin ligation is to regulate the function of Vav. Although the actual activity of Vav in SLP-76-deficient BMDCs following integrin ligation remains to be tested, impaired Vav function/localization could have dire consequences for the regulated activation of one or more of the Rho-family GTPases. Indeed, β₂ integrin-dependent activation of Rac1, Cdc42, and RhoA is impaired in neutrophils lacking Vav1 and Vav3 (37). Relatively little is known regarding the precise roles of the individual Rho GTPases in DCs following integrin ligation, although both Rac and Cdc42 have been shown to be important for optimal hemopoietic cell adhesion (38, 39). Specific effects of inhibiting Rac, Rho, and/or Cdc42 activity on cell morphology and podosome formation have also been described (40, 41). Interestingly, BMDCs deficient for Rac1 and Rac2 are severely impaired in the capacity to prime an Ag-specific T cell response, most likely due to an inability to interact with and form productive conjugates with T cells (42). Rac1/2-deficient DCs also show markedly reduced random motility on a fibronectin-coated coverslip in the absence of any chemotactic stimuli (42). Interestingly, we observe a remarkably similar phenotype (i.e., impaired random migration) using SLP-76-deficient BMDCs in a similar assay (N. Luckashenak, unpublished observations). Thus, measuring the activation and distribution of the Rac1/2 GTPases in the absence of SLP-76 may be particularly revealing. Another important possibility to consider is an alteration in the relative ratios of the activated GTPases in SLP-76-deficient DCs. Perhaps integrin-triggered signaling pathways that work through SLP-76 and Vav favor a specific temporal and/or activation pattern of Rho-family GTPases that is perturbed in the absence of SLP-76. Maintenance of this pattern may be critical for the capacity of DCs (and possibly additional hemopoietic cell types) to form dynamic cytoskeletal/adhesive structures that are more conducive to rapid changes in adhesion and migratory potential.

One of the most visually striking results obtained in the current study is the impact of SLP-76 deficiency on actin assembly and podosome distribution in BMDCs adhering to plate-bound RGDS peptide. Podosome formation is a regulated process requiring WASp activity, as DCs isolated from Wiskott-Aldrich syndrome patients or WASp-deficient mice fail to generate podosomes (40). In T lymphocytes, SLP-76 is required for the recruitment of Nck and consequently the localization of WASp to the TCR following receptor ligation (43). SLP-76 may therefore serve a similar role in DCs following integrin ligation. However, it is likely that WASp function is at least partly intact in SLP-76-deficient BMDCs, as the DC phenotype in WASp-deficient mice is more severe than that observed in the absence of SLP-76. Although the precise function of podosomes has remained elusive, they are thought to mediate adhesion and may represent active sites of matrix degradation and remodeling (33). In our experiments using wild-type BMDCs treated with LPS, podosome formation was evident within 10 min of contact with an RGDS-coated coverslip, and when observed these structures were consistently distributed in an even manner throughout the plane of contact between the adherent cell and the coverslip at all time points analyzed (up to 120 min). In marked contrast, podosomes forming in SLP-76-deficient BMDCs plated on poly(RGDS) were predominantly arranged in rosette-like structures reminiscent of those observed in developing chondrocytes and some transformed fibroblasts (35, 44). At this time, it is not clear whether this arrangement is directly related to aberrant or disrupted signaling, or more simply reflects a poorly adhesive state. It is important to note that previous studies have characterized the presence of podosomes primarily in immature DCs, with particular emphasis on podosomes forming at the leading edge of a polarized cell. Although mature DCs (e.g., LPS treated) generally produce fewer podosomes than immature DCs (40, 45), we were able to visualize podosomes in multiple DCs contained within our LPS-treated cultures. Still, it is possible that these cultures contained DCs at different stages of maturation, and that the DCs generating podosomes were not fully matured. We generally avoided using non-LPS-treated DCs in these studies, as these cells contained a high background level of constitutively phosphorylated SLP-76 and Vav. However, it remains of substantial interest to assess the impact of SLP-76 deficiency on podosome formation and distribution in non-LPS-treated BMDCs and freshly isolated primary DCs.

In addition to diminished phosphorylation of PLCγ2 and Vav, SLP-76-deficient DCs manifest a severe reduction in ERK1/2 phosphorylation following integrin ligation. Inhibition of ERK activity impairs chemotaxis in a variety of cell types on different substrata (46–49). The adaptor protein Paxillin, FAK, myosin L chain kinase, and calpain have each been implicated as substrates of ERK1/2 in response to integrin ligation (49–52). The defective phosphorylation of ERK1/2 in SLP-76-deficient DCs following integrin ligation could therefore impact directly the function of each of these proteins. The precise mechanism(s) governing
ERK1/2 activation following integrin receptor ligation, particularly in hematopoietic cells, is currently unknown. Studies in non-hematopoietic cells suggest the possibility that a signaling complex nucleated by FAK may activate ERK1/2 via Src and RAP-1-mediated activation of B-Raf, or through activation of MEK1 by the p21-activated kinase (53, 54). Whether or not SLP-76-mediated ERK1/2 phosphorylation and activation are completely independent of the FAK signaling cascade in hematopoietic cells remains to be determined. Our observation that ERK1/2 phosphorylation is markedly impaired in the absence of SLP-76 while FAK phosphorylation remains largely intact following integrin ligation suggests that a FAK-dependent pathway is not sufficient to compensate for the loss of SLP-76 in DCs.

Curiously, SLP-76-deficient DCs were observed to emigrate from ear tissue or through a Transwell insert (BMDCs) at an elevated frequency in response to the chemokine CCL21. Inhibition of ERK1/2 activity impairs DC chemotaxis in response to CCL21 (55) (N. Luckashenak, unpublished observations). However, we have found that CCL21-dependent phosphorylation of ERK1/2 remains largely intact in SLP-76-deficient BMDCs following exposure to CCL21 (data not shown), suggesting that this pathway is most likely functional in the absence of SLP-76. Still, it is not entirely clear why SLP-76 deficiency should result in enhanced migratory potential in these assays. In the ear explant model, suboptimal adhesion might be predicted to facilitate exit from the ear in response to a chemotactic stimuli. Presumably, integrin-dependent adhesion plays little role in the Transwell assay used in this study, suggesting that adhesion-independent migration may be enhanced in the absence of SLP-76. This would be consistent with the observation that Syk-deficient and Vav-deficient neutrophils manifest slightly elevated migration capacity and migratory speed (36, 37). Subsequent experiments will make use of Transwell inserts coated with one or more components of the extracellular matrix (e.g., fibronectin) or Matrigel as one means to more faithfully recapitulate the conditions faced by migratory DCs in situ.

Given the multiple defects in integrin-triggered signaling observed in SLP-76-deficient BMDCs, it is tempting to speculate that a number of in vivo DC functions may be compromised by the loss of SLP-76, including steady state localization in distinct areas of the secondary lymphoid tissues (e.g., Peyer's patches) and the ca-

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Disclosures
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