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Dendritic cells (DCs) are the most potent APCs for activating naive T cells, a process facilitated by the ability of immature DCs to mature and home to lymph nodes after encountering an inflammatory stimulus. Proteins involved in cytoskeletal rearrangement play an important role in regulating the adherence and motility of DCs. Vav1, a guanine nucleotide exchange factor for Rho family GTPases, mediates cytoskeletal rearrangement in hematopoietic cells following integrin ligation. We show that Vav1 is not required for the normal maturation of DCs in vitro; however, it is critical for DC binding to fibronectin and regulates the distribution but not the formation of podosomes. We also found that DC Vav1 was an important component of a signaling pathway involving focal adhesion kinase, phospholipase C-γ2, and ERK1/2 following integrin ligation. Surprisingly, Vav1−/− DCs had increased rates of migration in vivo compared with wild-type control DCs. In vitro findings show that the presence of adhesive substrates such as fibronectin resulted in inhibition of migration. However, there was less inhibition in the absence of Vav1. These findings suggest that DC migration is negatively regulated by adhesion and integrin-mediated signaling and that Vav1 has a central role in this process. The Journal of Immunology, 2009, 183: 310–318.

Vav1 Regulates the Migration and Adhesion of Dendritic Cells

David R. Spurrell,* Nancy A. Luckasenak,‡ Derek C. Minney,‡ Anna Chaplin,‡ Joseph M. Penninger,§ Robert S. Liwski,* James L. Clements,† and Kenneth A. West‡

Dendritic cells (DCs) are APCs that have a central role in activation of primary immune responses as well as maintenance of tolerance to self Ags (1, 2). Immature DCs reside in peripheral tissues where in response to inflammatory stimuli they acquire Ag and initiate a maturation process (3). DC maturation results in up-regulation of MHC class II and costimulatory molecules and migration of the DCs to lymph nodes LNs), where they present Ag to T cells. This movement of DCs from periphery to LN is critical for their function, as impairment in DC migration prevents productive T cell activation (4).

In response to external stimuli such as LPS and TNF-α, DCs undergo a tightly regulated differentiation that promotes migration. Immature DCs form strong attachments to the ECM primarily through the β1 integrins. With activation, DCs up-regulate chemokine receptors such as CCR7 that allow targeted homing to the LN. DCs must detach from ECM connections in peripheral tissues by down-regulating adhesive connections through integrins and cadherins (5, 6). Subsequent migration through ECM and into lymphatics requires cytoskeletal changes that regulate adherence and motility. Not surprisingly, several regulators of DC cytoskeletal rearrangement are involved in coordinating these responses. Rac, Rho, and Wiskott-Aldrich syndrome protein (WASP) are all implicated in the structural changes in DCs that occur during migration, and WASP-deficient DCs show impaired migration (7, 8). DCs lacking Rac1 and Rac2 fail to migrate in a random fashion, contributing to a reduced ability to engage and stimulate T cells in vitro (9). PI3Kγ is also critical for normal migration, suggesting at least one upstream pathway that may target the cytoskeleton and regulate DC migration (10). Recent work from our laboratory has demonstrated that the SLP-76 (Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa) adaptor protein is also required for optimal integrin-dependent adhesion and signaling in DCs (11). Indeed, integrins are now known to engage many of the same proximal signaling events as described for Ag receptors, including recruitment of ITAM-bearing accessory proteins (e.g., DAP12), activation of the Syk protein tyrosine kinase, and subsequent phosphorylation of the adaptor SLP-76 (12). Disruption of these pathways leads to marked defects in integrin-mediated function of platelets and neutrophils (13, 14). However, how these pathways are integrated and converge on downstream events in DCs (and hematopoietic cells in general) is just beginning to be explored.

Vav1 is a guanine exchange factor (GEF) for Rho family GTPases including Rac1, Rac2, and RhoA. Of the three Vav family members, Vav1 is unique in that it is selectively and ubiquitously expressed in cells of the hematopoietic lineage (15–17). DCs express Vav1 (18), although its role in DC function has not been extensively studied. Vav family GEFs are unique in their ability to directly link surface receptor signaling to actin cytoskeletal rearrangement. This is made possible by the presence of a DH homology (DH) domain and a Src homology 2 domain within the same

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4 Abbreviations used in this paper: DC, dendritic cell; FAK, focal adhesion kinase; GEF, guanine exchange factor; LC, Langerhans cell; LN, lymph node; PLC, phospholipase C; PLL, poly-L-lysine; PTK, protein tyrosine kinase; RGDS, Arg-Gly-Asp-Ser; SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa; WASP, Wiskott-Aldrich syndrome protein; WT, wild type.

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protein (reviewed by Hornstein et al. (19)). The single Src homology 2 domain mediates interactions with tyrosine-phosphorylated proteins in membrane signaling events (20). In T cells, Vav1 is activated following TCR ligation and binds phosphorylated tyrosine residues in the scaffold protein SLP-76 (21). This signaling pathway is critical for integrin-mediated adhesion in T cells (22–24) and neutrophils (25).

In this paper, we examined a role for Vav1 in regulating DC adhesion, migration, and cytoskeletal dynamics. Interestingly, despite markedly reduced adhesive properties and impaired integrin signaling potential, Vav1-deficient DCs demonstrated enhanced migration in vitro and homed to secondary lymphoid tissues with accelerated kinetics when compared with wild-type DCs. Surprisingly, despite marked defects in adhesion and adhesion-triggered signaling, the formation of actin-rich podosomes was not impaired by the absence of Vav1. These data indicate that Vav1-mediated signaling is required for coupling integrin ligation with more distal signaling events, and this may be important for negatively regulating DC migration.

Materials and Methods

Mice

C57BL/6 wild-type (WT) mice were purchased from Charles River Laboratories, and C57BL/6 Vav1−/− mice were a gift from Dr. J. Penninger (Institute of Molecular Pathology, Vienna Austria). All mice were housed at the Carleton Animal Care Facility (Dalhousie University, Halifax, Nova Scotia).

Abs and reagents

The following Abs were used for flow cytometry analysis: allophycocyanin anti-CD11c (eBioscience), PE anti-CD49e (BioLegend), PE anti-CD29 (BioLegend), anti-VLA-5 (Chemicon International), and PE-conjugated donkey anti-rat IgG (H+L) (eBioscience). Isotype controls included Armenian hamster IgG-FITC (BioLegend), rat IgG2a-PE (BioLegend), Armenian hamster IgG1-allophycocyanin (eBioscience), and rat IgG1-PE (eBioscience). For Western blotting, anti-Vav1, anti-Vav2, anti-Vav3 (all from Syrian hamster IgG-allophycocyanin (eBioscience), and rat IgG1-PE (eBioscience). Isotype controls included Ar-

Western blotting

SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk and incubated with the primary Abs overnight. Membranes were washed with PBS and incubated with the HRP-conjugated secondary Abs (H9262) and visualized using the WesternBlotting kit (Bio-Rad) or SuperSignal West Pico (Pierce) for protein visualization. Specific bands were quantified using ImageJ (NIH) software.

Flow cytometry

Expression of DC maturation markers and the fibronectin receptor on Vav1−/− and WT DCs was assessed at day 8 of culture following LPS stimulation and compared with that of WT DCs by FACS. DCs (5 × 10^6) were suspended in PBS and incubated with the appropriate Abs at 4°C for 45 min. Cells were washed twice with PBS and fixed in 1% paraformaldehyde. Fluorescence was analyzed for 10,000 cells per sample using a FACSCalibur flow cytometer (BD Biosciences), and the level of expression was evaluated using FCS Express V3 software.

Adhesion assays

Adhesion assays were performed in flat-bottom 96-well, nontissue culture-treated plates precoated with bovine fibronectin (20 μg/ml) or 1% BSA. Coated wells were blocked with 1% BSA at 37°C for 1 h. Mature bone marrow-derived DCs were harvested and rested in media without FCS at 37°C before plating. Cells (5 × 10^4) were added to the wells and allowed to adhere at 37°C. Nonadherent cells were removed by washing wells three to four times with media without FCS. The adherent cells were fixed in 2% paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich) for 25 min. Excess stain was removed by submersion in water, and cells were allowed to air-dry for 10 min. Stained cells were photographed before 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 and the OD was determined at 595 nm using a microtiter plate reader (Dynatech Laboratories).

Analysis of actin filament assembly and podosome formation

Glass coverslips were precoated with 15 μg/ml RGDS (Arg-Gly-Asp-Ser; Bachem) or 30 μg/ml fibronectin (Sigma-Aldrich) overnight at 4°C. The following day, LPS-treated DCs were harvested and rested in media without FCS at 37°C for 1 h. DCs (8 × 10^5) were adhered to glass coverslips for 45 min at 37°C. Coverslips were rinsed with PBS and adherent cells were fixed in 4% paraformaldehyde for 20 min. Cells were permeabilized in 0.5% Triton X-100 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 μg/ml coverslip). Some samples were co-stained with a vinculin-specific Ab (hV1N-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.

Integrin stimulation and immunoblotting

LPS-activated DCs were harvested and washed. Cells were rested in media without FCS at 37°C for up to 20 h and left in suspension or plated on 10-cm tissue culture-treated plates precoated with 10 μg/ml fibronectin, 15 μg/ml RGDS, and 0.01% poly-L-lysine (PLL). Adherent cells were collected and lysed at specific time points in 500 μl of RIPA buffer containing protease and phosphatase inhibitors. Western blots of whole-cell lysates were probed with Abs against phosphotyrosine (4G10; Upstate Biotechnology), phospho-p44/42 MAPK (E10), p44/42 MAPK, phospho-phospho- lipase C (PLIC)2, or PLCγ2, phosphorylated focal adhesion kinase (FAK), phospho-p38 MAPK or p38 MAPK, and moesin (Cell Signaling Technology). Appropriate HRP-conjugated secondary Abs (Jackson Immunoresearch Laboratories) were used in conjunction with a chemiluminescent substrate (SuperSignal West Pico; Pierce) for protein visualization. To resolve intracellular Vav1 proteins, DCs were lysed in hypotonic lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, and protease inhibitors). Protein concentrations were quantitated by Lowry (Bio-Rad). Twenty micrograms of protein per lane was resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blotted with the appropriate Abs overnight at 4°C in 5% milk in TBST and washed in 1× TBST before incubation with a HRP-labeled secondary Ab. After washing, the membranes were developed with ECL Plus (Amersham Biosciences).

In vivo migration assays

Left ears of Vav1−/− or WT mice were injected with 5 × 10^7 washed 1.0-μm fluorescein-conjugated, carbohydrate-modified microspheres (Molecular Probes) in 50 ng of TNF-α (28, 29). Right ears served as controls. Twenty-four hours later, the draining auricular LN were harvested, fixed in UMIFIX (Sakura Finetek) at 8 h at 4°C, and then transferred to 15% sucrose in PBS overnight. Tissues were frozen, cut into 20-μm sections on a cryostat (Leica Microsystems), and transferred onto sialinated slides. Tissue sections were blocked with 5% goat serum and incubated with the ER-TR7 Ab (Cedarlane Laboratories) overnight before adding the goat anti-rat Cy3 secondary Ab (Jackson Immunoresearch Laboratories). Images were captured using a Zeiss LSM 510 Meta confocal laser scanning microscope. The number of DCs was counted in 20 representative sections. In a second set of migration experiments mature DCs were generated from murine bone marrow of Vav1−/− or WT mice as described above. DCs were labeled with 5 μM CellTracker Green CMFDA (5-chlorometh-ylfluorescein diacetate) (Molecular Probes) for 30 min, washed, and then resuspended in PBS. DCs (2 × 10^6) in 50 μl of PBS were injected s.c. into each forelimb of WT mice. Twenty-four hours later, the draining auricular LN were harvested and processed as described above.

In vitro migration assays

Migration assays were performed in 24-well transwell plates (Costar) containing 5.0-μm inserts. The top and bottom of the membranes were coated overnight at 4°C with either PBS, 10 μg/ml fibronectin, or 0.5% BSA (fraction V; Sigma-Aldrich). Serum-free RPMI 1640 (600 μl) containing

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500 ng/ml CCL21 was added to each of the lower wells to serve as a chemoattractant. WT or Vav1−/− DCs (5.0 × 10^5) were added to the top chamber of each insert and incubated at 37°C. The cells that had migrated to the bottom chamber were counted using a hemocytometer. All migration conditions were done in triplicate and each experiment was repeated three times.

**Epidermal sheet preparation and in situ staining of Langerhans cells (LCs)**

To visualize LCs, left ears of WT and Vav1−/− mice were injected with 50 ng of TNF-α, while the right ear did not receive an injection and served as a control. After 4 h, the mouse was sacrificed and the ears were removed and placed in PBS. The dorsal and ventral ear halves were isolated and incubated at 37°C (dermal side down) for 20 min in 3.8% NH4SCN (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 an anti-Langerin (gift from Dr. S. Saeland, Schering-Plough Laboratory for Immunological Research, Dardilly, France) overnight at 4°C followed by goat anti-rat Cy3 for 1 h. Samples were photographed using an inverted fluorescent microscope equipped with a Nikon Eclipse TE300 camera. The numbers of LCs/mm² in the injected area were counted (30).

**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: *, p < 0.01; **, p < 0.001.

**Results**

**Vav1 is expressed in murine DCs**

Expression of Vav1 in human monocyte-derived DCs has been previously reported (18, 31). However, the role of Vav1 in DC function has not been described. We evaluated the expression of Vav1 in bone marrow-derived DCs by Western blot. Vav1 expression was readily detected in DCs cultured for 8 days, and it did not change appreciably following exposure to LPS (Fig. 1). Vav2 and Vav3 expression was observed in day 8 DCs, demonstrating that DCs express all three Vav isoforms (Fig. 1).

**Vav1 is not required for maturation of DCs in vitro**

We next sought to determine whether Vav1 was important during the differentiation and maturation of DCs from bone marrow cultures. Similar numbers of DCs were obtained from cultures of WT and Vav1−/− DCs (data not shown). DCs from Vav1−/− cultures demonstrated slightly enhanced maturation with a greater percentage of MHC class II-high DCs developing in Vav1−/− vs control cultures (23% vs 15%) after 8 days growth in GM-CSF. However, after treatment with LPS for 18 h there were no differences in percentage of CD11c<sup>high</sup>MHC-II<sup>high</sup> DCs (25% vs 23%) or in the mean level of expression of several maturation markers such as CD80 and CD86 (Fig. 2 A). Interestingly, unlike DCs deficient in other cytoskeletal regulatory proteins (9), Vav1−/− had normal

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**FIGURE 1.** Expression of Vav1, Vav2, and Vav3 proteins in C57BL/6 and Vav1−/− DCs. Vav1, Vav2, and Vav3 proteins were detected in C57BL/6 (lanes 1 and 2) and Vav1−/− DCs (lanes 3 and 4). Total cellular lysates from day 8 DCs treated with LPS (lanes 2 and 4) or media alone (lanes 1 and 3) were resolved by SDS-PAGE and detected by Western blot using Vav1-, Vav2-, or Vav3-specific Abs. An anti-β-actin Ab was used to demonstrate equal protein loading. Representative blots of three independent experiments are shown.

**FIGURE 2.** Vav1−/− bone marrow-derived DCs exhibit normal levels of maturation markers and morphology. A, Day 8 WT and Vav1−/− DCs were treated with LPS, and the expressions of CD11c, MHC class II, CD80, and CD86 were measured by flow cytometry. The isotype controls are shown as solid red lines, and specific Ab binding is shown in black. B, WT and Vav1−/− DCs were stained with anti-fascin Ab and analyzed by confocal microscopy. Results are representative of five independent experiments.
morphology with normal dendritic projections (Fig. 2B). This indicates that while Vav1 may play a minor role in early differentiation, there are no significant maturational abnormalities in terminally mature Vav1−/− DCs.

**Vav1 regulates adherence of DCs to fibronectin**

Vav1 is known to regulate T cell spreading on fibronectin (32), and previous work from our laboratory demonstrated that Vav1 was rapidly phosphorylated following integrin engagement (11). This suggested that Vav1 might be an important intermediate in the signaling pathways that govern integrin function in DCs. This led us to evaluate the binding of Vav1−/− DCs to a fibronectin-coated surface. WT DCs demonstrated much greater adherence to human and bovine fibronectin than did Vav1−/− DCs (Fig. 3, A and B). Indeed, adherence of Vav1−/− DCs to fibronectin was not different than adherence to BSA-treated culture plates, indicating that there was no specific adhesion to fibronectin. This poor adhesion was not due to alterations in integrin surface expression, as the levels of CD49e (fibronectin α5-chain), anti-CD29 (fibronectin β1-chain), or anti-VLA-5 (α5β1) were normal (Fig. 3C). Additionally, β2 integrin chain (CD18) and other β2 integrins such as CD11c and CD11b were similar in WT and Vav1−/− DCs (data not shown), indicating that impaired integrin function was more likely a consequence of impaired outside-in signaling than any receptor abnormalities.

**Alterations in cytoskeletal dynamics and podosome distribution in Vav1-deficient DCs**

Vav1 is a GEF for Rho family GTPases such as Rac1, Rac2, and Rho that mediate cytoskeletal rearrangement in other hematopoietic cells following integrin ligation. DCs along with other myeloid derived cells such as macrophages and osteoclasts form actin-rich adhesive structures known as podosomes (33). These consist of an F-actin core surrounded by a ring of integrins and other cytoskeleton-associated proteins such as vinculin. Podosomes are implicated in adhesion to extracellular matrix and play an important role in migration. In myeloid cells podosome number and size are regulated by cytoskeletal regulatory proteins such as WASP (34, 35) and Rac (36). We hypothesized that Vav1 might be an important regulator of podosomes in DCs. To examine the formation of actin-containing structures in DCs, we incubated WT or Vav1−/− DCs in plates precoated with fibronectin and then visualized F-actin-containing structures by confocal microscopy. Vav1−/− DCs had different morphology compared with WT, consistent with a poorly spread and loosely adherent phenotype. Abnormalities of DC morphological structure have also been seen in the absence of Rac (7, 9). To characterize podosome formation, vinculin and F-actin were visualized in DCs by confocal microscopy. Vav1−/− DCs had reduced numbers of podosomes compared with WT (Fig. 4). Unlike DC podosomes in SLP-76−/−, in Vav1−/− DCs the podosomes had a normal shape. However, the Vav1−/− podosomes were larger and had a more centralization/localized distribution. In macrophages, more localized podosomes are often seen with an activated migratory phenotype. Interestingly, macrophages expressing an active form of the Rho GTPase PAK4 have larger podosomes with a greater F-actin content, suggesting a complex regulation by interactions of multiple cytoskeletal regulatory proteins (37).

**Integrin signaling is altered in Vav1−/− DCs**

The poor adhesion and altered cytoskeletal dynamics observed in DCs lacking Vav1 suggested defective integrin signaling. Vav1 is an important mediator of integrin signaling events in platelets, T cells, and neutrophils. Activation of Vav1 GEF activity is mediated by its tyrosine phosphorylation (19). Previous work from our laboratory has also demonstrated that DC integrin ligation leads to phosphorylation of Vav1 (11). To begin to evaluate the Vav1-dependent integrin signaling pathways, we compared the global profile of tyrosine-phosphorylated proteins induced by integrin ligation in WT and Vav1−/− DCs. DCs were plated on RGDS-coated
wells for various time points, and whole protein lysates were subjected to Western blotting using an anti-phosphotyrosine Ab 4G10. WT DCs demonstrated early and prolonged tyrosine phosphorylation of several prominent proteins migrating at ~75 and 120 kDa (Fig. 5A). In contrast, there was a marked decrease in the levels of global tyrosine phosphorylation observed in Vav1−/− DCs. Interestingly, a very similar result was achieved using DCs lacking SLP-76, although the reduction in protein tyrosine phosphorylation was less profound when analyzing SLP-76−/− DCs (11). This suggests that SLP-76 and Vav1 function in a similar pathway downstream of integrin ligation that promotes optimal activation and/or localization of protein tyrosine kinases (PTKs) in DCs. However, the virtually complete absence of inducible tyrosine phosphorylation observed in DCs lacking Vav1 suggests that Vav1 may regulate additional, SLP-76-independent pathways that converge on PTKs. Therefore, Vav1 may influence the activation and localization of PTKs by several different pathways following integrin ligation.

To identify specific tyrosine phosphorylation events in DCs that may be affected by the absence of Vav1, we first focused on PLCγ2, as signaling complexes containing Vav1 are important for PLCγ2 phosphorylation following ITAM-mediated signaling, and it is recognized in other cell types, such as platelets, that the integrin and ITAM pathways employ similar signaling cascades. Both basal and integrin-stimulated PLCγ2 phosphorylation was markedly reduced in Vav1−/− DCs (Fig. 5B). Curiously, there was also a consistent inhibition of FAK phosphorylation on the position 397 tyrosine in the Vav1−/− DCs, demonstrating that Vav1 is important for activation of FAK (Fig. 5C).

Vav1 is necessary for integrin-dependent phosphorylation of ERK1/2 and p38 MAPK in DCs. Whole-cell lysates were prepared from WT or Vav1−/− DCs stimulated for the indicated times in wells precoated with poly-RGDS or PLL. Nonadherent cells (suspension) were used as a negative control. Proteins were resolved by SDS-PAGE and immunoblotted with Abs for ERK1/2 and p38 MAPK. S, suspension; P, PLL; R, RGDS. Representative blot is shown.

Vav1 is required for integrin-dependent activation of ERK1/2 MAPKs in DCs

MAPKs mediate multiple downstream events including gene transcription and cytoskeletal dynamics that result in functional changes in the cell following integrin signaling. In NK cells, Vav1 is required for integrin-dependent phosphorylation of ERK1/2 and p38 MAPK (38, 39). Therefore, we examined a role for Vav1 in ERK1/2 and p38 MAPK activation in DCs. In WT DCs plated on PLL or RGDS there was an early increase in ERK1/2 phosphorylation that decreased with time. While PLL is not considered a direct integrin agonist, PLL-triggered adhesion clearly initiates some intracellular signaling events sufficient for PTK activation and ERK1/2 phosphorylation. In contrast, p38 phosphorylation was only observed in WT DCs following integrin-mediated signaling (RGDS), and like the ERK1/2 phosphorylation was transient in nature. In the absence of Vav1 there was a marked reduction in the phosphorylation of ERK1/2 and p38 under all conditions that promote adhesion (Fig. 6). This demonstrates that
Vav1 is important in mediating adhesion-dependent signaling events required for optimal phosphorylation (and likely activation) of several MAPK family members in DCs.

**Vav1−/− DCs have defects in integrin attachment and signaling**; therefore, we evaluated migration of Vav1−/− DCs to LNs in vivo.

**FIGURE 7.** Vav1−/− DCs have enhanced migration to LNs. The in vivo migratory capacity of DCs was evaluated by injecting WT or Vav1−/− mice with fluorescein-conjugated carboxylate-modified microspheres (A and B) or injecting WT or Vav1−/− DCs prelabeled with CellTracker Green CMFDA into WT mice (C and D). Draining LNs were harvested at various time points and LN sections were stained with ER-TR7 to identify fibroblastic reticular cells and examined by confocal microscopy. To quantitate the number of DCs migrating to LNs, the numbers of beads (B) or cells (C) in 20 LN sections were determined and the average numbers of migrating cells per section were calculated. D, Representative images of WT or Vav1−/− DCs stained with CellTracker Green CMFDA and injected s.c. Results are representative of four independent experiments.

Vav1 is important in mediating adhesion-dependent signaling events required for optimal phosphorylation (and likely activation) of several MAPK family members in DCs.

**Vav1−/− DCs migrate to LNs with enhanced kinetics**

DC migration from the periphery to the draining LN is critical for the induction of primary immune responses. Migration is dependent on cytoskeletal rearrangement, and several cytoskeletal regulatory proteins, including WASP, Cdc42, and PI3Kγ, have been implicated in regulating DC migration (8, 10, 40). Interestingly, DCs deficient in some of these regulatory proteins show impaired podosome formation in addition to altered migration dynamics (7). Vav1−/− DCs have defects in integrin attachment and signaling; therefore, we evaluated migration of Vav1−/− DCs to LNs in vivo.
DC migration can be studied in vivo using s.c. injected fluorescent microspheres that are trafficked to LNs inside of DCs (28, 29). Therefore, we injected WT or Vav1−/− mice s.c. with FITC beads. LNs were collected at various time points, sectioned, and stained with ER-TR7 (to visualize the structural fibroblastic reticulum cells of the LN). LN sections were examined by confocal microscopy and the number of beads in each section was counted. Surprisingly, at 24 h postinjection, significantly more Vav1−/− cells were present in the LN as compared with WT DCs, indicating that there was accelerated migration of DCs in Vav1−/− animals (Fig. 7, A and B). However, at later time points fewer Vav1−/− DCs were present in LNs. This suggested that Vav1 deficiency also altered the persistence of DCs in LNs, although we cannot formally conclude that this is the case. No visible difference was seen in the distribution of DCs in the LN. Although this approach demonstrated that endogenous DCs migrate faster in Vav1−/− animals, it did not allow us to distinguish whether the enhanced migration was due to factors extrinsic to the DCs. To address these issues WT or Vav1−/− bone marrow-derived DCs were generated, labeled with green CMFDA dye, and injected into the forelimb of WT mice. Consistent with the bead injection approach, there were increased numbers of Vav1−/− DCs in the LN 24 h following injection and again decreased numbers of Vav1−/− DCs compared with WT at 48 h postinjection, thus confirming that Vav1 normally influences the migration rate of DCs in vivo (Fig. 7, C and D).

Enhanced migration of Vav1−/− LCs following activation

Altered DC migration dynamics could occur due to an accelerated ability to emigrate from peripheral sites, more rapid transit through lymphatics, an enhanced ability to enter the LN, or some combination of effects. We next investigated whether the enhanced DC migration seen in the absence of Vav1 altered emigration from skin. We injected the right ears of both Vav1−/− and WT mice with a TNF-α stimulus with the left ear serving as a control. In normal mice there was a decrease in the density of LCs from 800/mm² to 560/mm² after 4 h (Fig. 8A). There was a much more pronounced change in the Vav1−/− mice where the density of LCs decreased from 840/mm² to 400/mm² (Fig. 8).

Vav1−/− DCs have an increased migratory capacity in vitro

Since Vav1−/− DCs had an increased rate of migration to LNs, we decided to examine if this could be recapitulated in vitro. To evaluate this, we used a standard transwell migration assay in which the inserts were coated with PBS, fibronectin, or BSA. The ability of day 8, LPS-treated DCs to migrate toward CCL21 (in the bottom chamber) was then measured. Vav1−/− DCs clearly had an increased migratory pattern toward CCL21 in all three groups tested (Fig. 9). When wells were coated with fibronectin, there was a greater relative decrease in WT DC migration as compared with the Vav1−/− DCs, indicating that adhesion to fibronectin normally influences the rate of DC migration, and that decreased adhesion (as seen with Vav1−/− DCs) results in enhanced migration rate.

Discussion

This study provides the first evidence that Vav1 is an important regulator of integrin-mediated adhesion and migration in DCs. DCs express Vav1, Vav2, and Vav3; however, we demonstrated that Vav1 has a nonredundant role in DC function. Vav1-deficient DCs underwent normal maturation and development and expressed normal levels of α5β1 integrin. Vav1 was critical for the binding of DC to fibronectin. While Vav1 was dispensable for the formation of podosomes, it influenced their size and distribution. We demonstrated that Vav1 was a critical component of the signaling pathway that activates FAK and ERK following integrin ligation. Somewhat surprisingly, we found that Vav1−/− DCs migrated at enhanced rates to LN in vivo in both mutant and WT mice, indicating that Vav1 regulates pathways influencing the kinetics of migration. Consistent with the in vivo migration data, a higher number of Vav1-deficient DCs migrated through fibronectin-coated transwell inserts when compared with WT DCs during the same time, most likely due to decreased adhesion and shorter contact time with the substratum.
Our observation that Vav1 is important for the optimal adhesion of DCs to fibronectin is consistent with previous reports demonstrating that Vav1 is involved in the binding and spreading of T lymphocytes on fibronectin (32). Also, Vav1 phosphorylation on Y160 after integrin ligation is necessary for cytoskeletal reorganization and cell adhesion (41). Similarly, we recently demonstrated that DCs deficient in SLP-76 are also impaired in their integrin-dependent adhesion to fibronectin (11). Both Vav1 and SLP-76 undergo phosphorylation following α5β1 integrin ligation, suggesting that Vav1 and SLP-76 likely function as part of a signaling complex downstream of α5β1 integrin in DCs. This pathway subsequently mediates PLCγ2 activation similar to that which is seen in T cells (42).

Furthermore, defective phosphorylation of ERK1/2 is seen in both Vav1 and SLP-76-deficient DCs plating in fibronectin-coated tissue culture wells. In other cell types, FAK, paxillin, and calpain have been implicated as substrates of ERK1/2 in response to integrin ligand (43–45). Conversely, in nonhematopoietic cells FAK may activate ERK1/2 via a B-Raf-dependent pathway (46). However, we have shown that FAK activation remains intact in SLP-76−/− DCs but not in Vav1−/− DCs, suggesting that in DCs, FAK and ERK1/2 activation may remain in two distinct signaling pathways.

Despite marked defects in integrin signaling and adhesion, Vav1−/− DCs clearly generated podosome-like structures upon adhesion to a fibronectin- or RGDS-coated substratum. Podosomes are cone-shaped structures consisting of a central area of actin and β1 integrins that form on the bottom surface of migrating cells such as DCs, macrophages, and osteoclasts (33, 47, 48). Rapid actin turnover is found in podosomes (49), and formation and turnover of podosomes are regulated by a number of cytoskeletal regulatory proteins. WASP-deficient DCs and macrophages are unable to form podosomes and have marked abnormalities in migration (7). Other regulatory proteins such as Cdc42, Rac, and Rho also contribute to the generation of podosomes (7), although the exact signaling pathways have not been determined. While podosome-like structures containing an obvious actin core surrounded by vinculin were visible in Vav1-deficient DCs, further studies are required to determine whether these podosomes contain the full complement of signaling and adaptor proteins that may confer podosome function.

The relationship between podosomes and migration is complex. Immature DCs form podosomes, but during maturation, podosomes disappear as endocytosis proceeds. As DCs mature further and start to migrate, podosomes are reformed (49). PGE2, also results in rapid dissolution of podosomes and the development of a high-motility DC (5). These authors did not find that podosomes re-formed in mature migrating DCs. Interestingly, while the podosomes that formed in the Vav1−/− DCs were larger, we found that those formed in SLP-76−/− DCs were present in rosette-like structures similar to those observed in src-transformed cells (11). Thus, both SLP-76 and Vav1 likely function to coordinate β1 integrin signaling events in DCs that ultimately regulate podosome dynamics. However, the podosome “phenotype” for each strain of mutant mice is quite distinct, indicating that SLP-76 and Vav1 do not function in one linear pathway that targets a common set of downstream mediators.

We were quite surprised that Vav1-deficient DCs demonstrated faster migration to LNs in vivo. Our initial study evaluated the migration of skin DCs in intact Vav1−/− mice using fluorescent latex beads. We confirmed that the enhanced migration of Vav1-deficient DCs using this system was due to an intrinsic property of the Vav1−/− DCs and not a consequence of alterations in the surrounding tissues or other lymphoid components by injecting Vav1−/− DCs into WT mice and monitoring subsequent homing to the draining LN. These data correlated well with an increase in emigration of Vav1-deficient LCs from skin in response to cytokine stimuli (Fig. 8). Collectively, our data suggest that Vav1 normally regulates signals that impact directly on the rate of migration, most likely by promoting adhesion. In the absence of Vav1, the reduced adhesive potential translates into enhanced migration, which we observed both in vitro and in vivo. Interestingly, a recent study demonstrated that integrins (and presumably integrin signaling) are dispensable for the in vivo migration of DCs to LNs (50).

The role of Vav1 in regulating migration in other cells is less clear and has been predominantly examined in vitro. In macrophages, deficiency of Vav1 results in a reduced migration speed but this was evaluated in vitro (51). In SHIP−/− macrophages Vav1 is constitutively active and it is recruited to the membrane in a PI3K-dependent manner. Vav1 inhibition inhibited chemotaxis and expression of constitutively active onco-Vav increased migration (52). Overexpressing Vav1 impaired migration of peripheral blood lymphocytes, but in the same system a dominant-negative version of Vav1 also impaired migration (53). The authors raised the interesting observation that dominant negatives of Vav1 that impair migration may bind to the downstream mediators of Vav and prevent other forms of Vav from taking over and being redundant. This may suggest that Vav1 functions to impair migration while Vav2 or Vav3 enhance migration.

Other molecules in the integrin signaling pathway have been demonstrated to inhibit migration in leukocytes. Our laboratory has previously demonstrated that DCs deficient in SLP-76 also have impaired integrin-mediated binding and demonstrate enhanced migration in vitro and in vivo (11). We also identified that SLP-76 is phosphorylated and associates with Vav1 following integrin ligation, indicating that they are likely acting in the same integrin-mediated signaling pathway. In neutrophils Syk was important for integrin-dependent signaling and neutrophil adhesion. Syk-deficient neutrophils migrated faster in vitro and in vivo into inflamed peritoneum (54). Our current data extend these findings and suggest that in DCs there is a reciprocal relationship between integrin-mediated signaling and DC migration.

DC maturation is tightly regulated in a temporal fashion and it is likely that altered DC migration dynamics could alter subsequent T cell activation. Integrin signaling may slow the arrival of DCs in the node, allowing appropriate maturation to occur. Different T cell outcomes may be expected if T cells were responding to incompletely matured DCs. Premature arrival of the DCs in the LN could result in a disruption of the balance between activation of regulatory and effector T cells (55). Alternatively, the cytokine profile of DCs varies dramatically in the 48 h following activation (56). Early following activation DCs trigger Th1 responses, whereas at later time points they induce Th2 responses. The earlier arrival and departure of integrin signaling-deficient DCs in the LN may result in a predominant Th1 response. Future studies in the area of integrin signaling and DC migration will need to focus on the relationship between the kinetics of migration and the ensuing T cell response.

Disclosures

The authors have no financial conflicts of interest.

References

dissolution and induction of high-speed migration during dendritic cell maturation.


