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*J Immunol* 2011; 186:2850-2859; Prepublished online 24 January 2011;
doi: 10.4049/jimmunol.1001667
http://www.jimmunol.org/content/186/5/2850

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/01/24/jimmunol.1001667.DC1

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Fascin1 Promotes Cell Migration of Mature Dendritic Cells

Yoshihiko Yamakita,* Fumio Matsumura,* Michael W. Lipscomb,† Po-chien Chou,‡ Guy Werlen,§ Janis K. Burkhardt,† and Shigeko Yamashiro*

Dendritic cells (DCs) play central roles in innate and adaptive immunity. Upon maturation, DCs assemble numerous veil-like membrane protrusions, disassemble podosomes, and travel from the peripheral tissues to lymph nodes to present Ags to T cells. These alterations in morphology and motility are closely linked to the primary function of DCs, Ag presentation. However, it is unclear how and what cytoskeletal proteins control maturation-associated alterations, in particular, the change in cell migration. Fascin1, an actin-bundling protein, is specifically and greatly induced upon maturation, suggesting a unique role for fascin1 in mature DCs. To determine the physiological roles of fascin1, we characterized bone marrow-derived, mature DCs from fascin1 knockout mice. We found that fascin1 is critical for cell migration: fascin1-null DCs exhibit severely decreased membrane protrusive activity. Importantly, fascin1-null DCs have lower chemotactic activity toward CCL19 (a chemokine for mature DCs) in vitro, and in vivo, Langherans cells show reduced emigration into draining lymph nodes. Morphologically, fascin1-null mature DCs are flatter and fail to disassemble podosomes, a specialized structure for cell-matrix adhesion. Expression of exogenous fascin1 in fascin1-null DCs rescues the defects in membrane protrusive activity, as well as in podosome disassembly. These results indicate that fascin1 positively regulates migration of mature DCs into lymph nodes, most likely by increasing dynamics of membrane protrusions, as well as by disassembling podosomes. The Journal of Immunology, 2011, 186: 2850–2859.

Upon maturation, dendritic cells (DCs) change their functions from Ag sampling to Ag presentation, in a process involving massive alterations in their morphology and motility. The actin cytoskeleton plays key roles in multiple aspects of DC function (1–5). For example, Ag presentation by mature DCs depends on the integrity of the actin cytoskeleton (5). Rac1/2, a small G protein that is responsible for ruffling movements, has been shown to be essential for the interaction of DCs with T cells (1). Wiskott-Aldrich Syndrome protein, a molecule that controls Arp2/3-dependent actin polymerization, is required for the formation of the immunological synapse (2, 6). Although there is abundant evidence that alterations in actin dynamics are important for maturation-associated changes in DCs, it is not clear which actin regulatory proteins induce the profound alterations in morphology and motility observed upon DC maturation. Rac and Cdc42, which control the assembly of membrane ruffling and filopodia, respectively (7), are unlikely to play major roles in these alterations. Rac activity appears to be either unchanged or increased minimally upon maturation (8, 9), whereas Cdc42 activity is decreased upon maturation (9).

In addition to the profound alterations in morphology and motility, DCs show reduced adhesion to the substrate upon maturation, as evidenced by the loss of podosomes, specialized structures for cell-matrix adhesion (4, 10–12). Podosomes consist of an actin core surrounded by a characteristic ring structure containing adhesion molecules, including vinculin, talin, paxillin, and integrin (13, 14). DCs transiently lose podosomes at ~20 min after activation with LPS, then recover podosomes by 2 h. Later, they permanently lose podosomes at ~5–7 h after activation, concomitant with the generation of characteristic veil-like membrane protrusions (4, 10, 11). Although the first and transient loss of podosomes is controlled via pathways involving PGE2, RhoA, and rho-kinase (10, 15), as well as ADAM17 (12), it is not clear what causes the second and permanent loss of podosomes in mature DCs.

Fascin1, an actin-bundling protein (see for review Ref. 16), is specifically induced to a great extent upon DC maturation, whereas it is not detectable in immature DCs (17–20). Other blood cells such as primary macrophages and T cells do not express or induce fascin1. The induction of fascin1 in DCs suggests a specific role for fascin1 in DC maturation. Fascin1 has been suggested to be critical for assembly of filopodia or membrane protrusions. Fascin1 is localized to filopodia (21–25), and fascin1 overexpression induces membrane protrusions and increases cell motility of epithelial cells and colon carcinomas (26–28). Conversely, fascin1 knockdown has been reported to block assembly of filopodia in cultured mammalian cells (27–29). In bone marrow–derived DCs, fascin1 depletion by antisense treatment resulted in the inhibition of both dendrite formation and T cell activation (19, 20, 30). However, little is known about what roles fascin1 plays in motility and cytoskeletal reorganization associated with DC maturation.

To determine the physiological function of fascin1 in DC maturation, we generated fascin1 knockout (KO) mice (31). Fascin1 KO mice provide an excellent experimental system, allowing...
us to analyze fascin function in DCs with little experimental manipulation. In addition, fascin KO mice provide fascin-null phenotypes that are complete and uniform, unlike antisense or small interfering RNA approaches that tend to be partial and variable. Our results indicate that fascin1 profoundly alters DC cytoskeletal organization and plays critical roles in migration of mature DCs into lymph nodes for Ag presentation.

Materials and Methods

Reagents, Abs, and fascin1-deficient mice

The following Abs were used: FITC-conjugated hamster anti-mouse CD11c mAb (BD Biosciences, San Diego, CA); FITC-conjugated rat anti-mouse CD11c (55k-2) mAb; FITC-conjugated rat anti-CD11c (RT-2) mAb. R-PE-conjugated 1-AF-E (MHC-II) mAb (BD Biosciences); mouse anti-vinculin mAb (Sigma-Aldrich, St. Louis MO); rabbit anti-α-actinin Ab (25); mouse anti-fascin mouse mAb (55k-2) (25); and chick anti-fascin Ab (generated by Aves Laboratories [Tigard, OR], using recombinant human fascin as an Ag). GM-CSF and CCL19 (MIP3β) were purchased from Invitrogen (Carrollton, CA).

Fascin1 KO heterozygous mice were generated by Lexicon Pharmaceuticals (Woodlands, TX) from an embryonic stem cell line (OST124903; Lexicon’s OmnibusBank library of gene KO embryonic stem cell clones), and backcrossed with C57BL/6 female mice for >16 generations (31). Both RT-PCR and Western blot analyses revealed that the loss of fascin1 is not compensated with the expression of the fascin1 paralogs (such as retina fascin2 and testis fascin3) (31). For each experiment with homozygous mice, their wild-type littermates were used as a control. All experimental procedures and protocols for mice are approved by the Animal Care and Facilities Committee at Rutgers. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at Rutgers.

Preparation of bone marrow DCs

Preparation of mouse bone marrow DCs was according to the method described in Inaba et al. (32), with slight modification. Briefly, single-cell suspension was prepared from bone marrow of femurs and tibias, and plated on 65-mm dishes in DMEM containing 10% FCS and 10 ng/ml GM-CSF for 7–10 d. Nonadherent cells were collected and DCs were purified by centrifugation over a 13.7% (w/v) metrizamide discontinuous gradient. More than 85% of cells collected at the interface of the gradient were positive for CD11c. Cells were matured by overnight culture in the presence of 100 ng/ml LPS (Sigma-Aldrich).

FACS analyses

Mature DCs were fixed with methanol or formalin, and stained with FITC-labeled anti-DC markers including CD86, CD11c, and MHC-II. For double labeling, methanol-fixed cells were blocked with a rat anti-mouse CD11c/CD32 Ab (mouse Fc block; BD Pharmingen) and incubated with the mouse anti-fascin1 Ab (clone 55k-2) together with the FITC-labeled CD86 Ab, and then the fascin Ab was labeled with a R-PE-labeled goat anti-mouse IgG. Flow cytometry was performed with a Coulter Cytomics FC500 flow cytometer.

Immunofluorescent microscopy and measurements of thickness, area, and circularity

For staining with Abs against CD11c, CD86, MHC-II, and vinculin, as well as for staining with rhodamine phalloidin (Molecular Probes, Eugene, OR), DCs were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100 or 100% acetone. Absolute methanol fixation at −20°C was used for double labeling with the anti-fascin1 mouse mAb (clone 55k-2) and the anti-CD86 Ab, and for double staining with anti-fascin1 and anti-α-actinin Abs. Images were taken as Z-stacks (0.2 μm spacing) with a DeltaVision Image Restoration Microscope system (Applied Precision, Issaquah, WA), deconvolved either with the softWoRx software (Applied Precision) or the Huygens software (Scientific Volume Imaging, Hilversum, The Netherlands). Projected images were generated with SoftWoRx or ImageJ (http://rsb.info.nih.gov/ij/). In some experiments, images were taken on a Nikon TE300 microscope with a ×60 objective lens (NA 1.4). Exposure times for imaging and settings for deconvolution were constant for all samples to be compared within any given experiment. For presentation, images were cropped and brightness were adjusted with Photoshop (Adobe, San Jose, CA).

For measurements of thickness, area, and circularity, wild-type and fascin1 KO DCs were labeled with the FITC-labeled CD86 Ab, rhodamine phalloidin, and DAPI. Because the expression of CD86 is well correlated with that of fascin1 (see FACS analyses shown in Fig. 1A), CD86mKO DCs were chosen to compare differences in thickness, area, and circularity between fascin1-expressing wild-type and fascin1-null DCs. Orthogonal images created by SoftWoRx were used for measurement of thickness. Areas were measured with yl images of DCs at the ventral focal plane, and circularities were measured with Z-projected images. Both areas and circularities were measured using ImageJ software.

Live cell imaging, kymography, microinjection, and transfection

For phase-contrast, live cell imaging, DCs were placed at 37°C in a temperature-controlled incubator (MS200D; Narishige) and observed under a Nikon microscope (TE300 with a ×40 Plan Fluor phase-contrast (NA 0.60) objective lens. Time-lapse images were taken every 10 s for 20–30 min by a CCD camera (CoolSnap-fx; Roper Scientific) with IPLab image analysis software (Scanalytics). Two to three kymographs were generated for each cell with randomly chosen, one-pixel lines using ImageJ (National Institutes of Health) with the Kymograph plug-in (written by J. Rietdorf and A. Seitz, European Molecular Biology Laboratory). Kymographs were then analyzed using ImageJ to determine rates of membrane protrusions and retractions.

Microinjection of GFP fascin1 into differentiated THP-1 (human acute monocytic leukemia cell line) cells was performed as follows: cells were first differentiated into macrophages by the treatment of 200 nM 2-O-tetradecanoylphorbol-13-acetate for overnight, as described (33). Micro-injection was performed as described previously (26), using GFP fascin1 at a needle concentration of 9 ng/ml. As a control, FITC-labeled BSA was injected. After 1-h incubation, cells were fixed with formaldehde, permeabilized with acetone, and counterstained with rhodamine-labeled phalloidin or the anti-vinculin Ab to determine effects on podosome assembly. To estimate levels of fascin1 in injected cells, injected cells were stained with the fascin1 Ab, and fluorescent intensities were compared with those of uninjected cells and fascin1-expressing DCs. It was found that injection increased the fascin1 level 5–10 times over the level of endogenous fascin1 in THP-1 cells, the level of which is comparable to that found in wild-type DCs.

Mature or immature DCs were transfected with a human GFP fascin1 fusion construct (26) using an Amaxa Nucleofector II, according to the manufacturer’s instructions.

In vitro assay for chemotaxis

Chemotaxis of mature DCs was assayed in triplicate using a modified Boyden chamber assay. CCL19 (MIP3β), a chemokine for mature DCs, was added in bottom wells at the concentration of 0.6 μg/ml. Mature wild-type and fascin1 KO DCs (2 × 105 cells) were placed on top wells of a collagen-coated Boyden chamber with 3-μm hole (Corning, Lowell, MA). After 24-h incubation, cells transmigrated into the bottom chamber were counted.

Preparation of epidermal sheets and assay for DC migration into lymph nodes

Epidermal sheets before and after stimulation by FITC painting were stained essentially as described (34). Briefly, the dorsal surfaces of both ears of wild-type and fascin1 KO mice were painted with 25 μl 1% FITC in acetone/ dibutylphthalate (1:1). After 24 h, epidermal sheets were isolated from dorsal halves of the ears and stained with anti–MHC-II Ab, followed by Cy3-labeled donkey anti-rat Ab. Fifteen to 20 randomly selected fields were photographed with a Nikon TE300 with a ×20 objective, and the number of Langerhans cells per field (area, 149,000 μm2) was counted.

Langerhans cells migrated into draining lymph nodes were prepared essentially as described (35, 36). Briefly, mice were painted with the FITC solution, as described above, except that both dorsal and ventral sides of the ears were painted with 15 μl solution (total 30 μl per one ear). After 24 h, draining lymph nodes (auricular) were excised and cell suspension was prepared through cell strainers (Falcon, 70 μm). DCs were then enriched on metrizamide discontinuous gradients, as described above, and cytopun onto coverslips. FITC-bearing DCs were identified and counted by fluorescence microscopy or phase-contrast microscopy, and Langerhans cell migration was expressed as numbers of FITC-bearing DCs per lymph node.

Scanning electron microscopy

DCs grown on coverslips were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 20 min at room temperature, and dehydrated...
by submerging graded ethanol solutions. After critical point drying and platinum coating, images were taken with an Amray 1930I scanning microscope.

Statistical analysis
Statistical analyses were performed using Student t test (http://www.physics.csbsju.edu/stats/t-test.html).

Results
Fascin1 deficiency does not alter expression of DC maturation markers or other actin-binding proteins
As a first step toward characterization of fascin1-null DCs, FACS analyses were performed to determine whether fascin1 deficiency affects expression of DC markers, including CD11c, CD86, and MHC-II, when bone marrow-derived DCs were activated by 100 ng/ml LPS. As shown in Fig. 1A, surface expression of the DC marker CD11c (Fig. 1Aa) and the maturation markers MHC-II and CD86 (Fig. 1Ab, 1Ac) were similar between wild-type and fascin-null, mature DCs, indicating that fascin1-null DCs are able to mature in terms of the expression of maturation markers. As expected, fascin1 was expressed only in wild-type, but not fascin1 KO DCs (Fig. 1Ad, 1Af). As shown in Fig. 1Ae, double labeling with anti-fascin1 and anti-CD86 Abs revealed a strong correlation between fascin1 and CD86 expression in wild-type DCs. Before LPS activation, both wild-type and fascin1-null DCs expressed similar levels of CD11c, whereas neither cell type expressed appreciable levels of CD86 (data not shown). These results are consistent with previous reports that fascin1 depletion by antisense treatments did not alter expression of DC maturation markers (19, 20, 30).

Western blot analyses confirmed that fascin1 is induced upon maturation of wild-type DCs (Fig. 1B, lane 2), whereas it is absent in immature, wild-type DCs (lane 1), as well as immature and mature fascin1 KO DCs (lanes 3 and 4, respectively). We examined whether fascin1 deficiency alters expression of other actin-bundling proteins, including fimbrin and α-actinin. Fimbrin levels are largely unaltered between wild-type and fascin1 KO DCs, regardless of maturation. α-Actinin was barely detectable in DCs, and its levels appear unchanged. The levels of vinculin, however, are slightly increased in fascin1 KO DCs.

Fig. 1C shows immunofluorescence localization of fascin1 and CD86 of wild-type as well as fascin1 KO DCs. As expected, fascin1 KO DCs showed no fascin1 staining, whereas CD86-staining intensities were similar between wild-type and fascin1 KO DCs, confirming the FACS analyses. As previously reported (17, 25, 37, 38), fascin1 was localized at filopodia-like structures.

Fascin1 is present in the cortex of membrane protrusions
We found that fascin1 was also localized at the cortex of veil-like membranes (a more prominent structure than filopodia in mature DCs) of mature wild-type DCs. Fig. 1D shows wild-type and fascin1 KO DCs double labeled with phalloidin and anti-fascin1 Ab and imaged at two different focal planes (the ventral surface and a middle). In wild-type DCs expressing high levels of fascin1, both fascin1 and F-actin were colocalized at the cortex of veil-like protrusions (arrowheads in Fig. 1Da–f). In contrast, fascin1 KO DCs were more spread and showed many fewer veil-like protrusions either at the ventral surface or at the middle focal plane. In addition, fascin1 KO DCs frequently showed a cluster of many prominent actin dots at the ventral surface, which were reminiscent of podosomes (arrow in Fig. 1Dh). In contrast, most wild-type DCs did not exhibit such large and clustered dots. As described later, vinculin labeling revealed that these dots observed in fascin1 KO DCs indeed represent podosomes (see Fig. 2).

Fascin1 is essential for dynamics of membrane protrusions
The presence of fascin1 in the cortex of membrane protrusions (Fig. 1D) prompted us to examine whether the dynamics of membrane protrusions are altered by fascin1 deficiency. To test this, we imaged live DCs plated on glass coverslips using phase-contrast microscopy (Supplemental Videos 1, 2 for wild-type DCs, Supplemental Videos 3–5 for fascin1-deficient DCs). Fig. 3A–C illustrates representative still images of wild-type (A, corresponds to Supplemental Video 1) and fascin1-null (B and C, corresponds to Supplemental Video 3, 4, respectively) DCs, respectively. Fig. 3B represents the majority of fascin1-null DCs, whereas Fig. 3C represents a minor fraction (<20%) of fascin1-null DCs. These images clearly demonstrate that membrane activity of wild-type DCs is much more vigorous than that in fascin-null DCs. Kymograph analyses (Fig. 3D–F) confirm that fascin1-null DCs (Fig. 3E, 3F) displayed greatly diminished dynamics compared with wild-type DCs (Fig. 3D). Box plot analyses of protrusion (Fig. 3G) and retraction (Fig. 3H) rates generated from kymographs (representing seven different live cell imaging experiments) revealed that the median value of the protrusion rate for wild-type DCs (0.11 μm/s, n = 83) was 2.1 times higher than that of fascin1-deficient DCs (0.053 μm/s, n = 85), and the retraction rate in wild-type DCs (0.10 μm/s, n = 84) was 4 times higher than that of fascin1-deficient DCs (0.025 μm/s, n = 87).

To determine whether fascin1 is responsible for the dynamic membrane movements, we re-expressed GFP fascin1 in fascin1 KO DCs, and tested whether fascin1 was able to rescue the poor dynamics of membrane protrusions. Phase-contrast, time-lapse microscopy was performed with fascin1 KO DCs expressing GFP alone (control, see Supplemental Video 6) or GFP fascin1 (Supplemental Video 7). As shown in both still images (Fig. 3I, 3J) and kymographs (Fig. 3K, 3L), DCs expressing GFP fascin1 (Fig. 3J, 3L) exhibited much greater membrane protrusion dynamics than did DCs expressing control GFP (Fig. 3I, 3K). Box plot analyses (Fig. 3M, 3N) revealed a significant difference in both protrusion and retraction rates (p < 0.0001): the median protrusion and retraction rates for DCs expressing GFP fascin1 were 0.18 μm/s (n = 103) and 0.13 μm/s (n = 98), respectively, whereas those of DCs expressing GFP were 0.058 μm/s (n = 74) and 0.043 μm/s (n = 50), respectively. Thus, the protrusion/retraction rates of GFP fascin1-expressing DCs were comparable to those of wild-type DCs, whereas those for GFP-expressing DCs were similar to those of fascin1-deficient DCs. These results indicate that fascin1 induction upon maturation is responsible for the vigorous dynamic membrane movements of mature DCs.

Fascin1 is important for in vitro chemotaxis of mature DCs toward CCL19
The lower membrane protrusive activity of fascin1-deficient, mature DCs would be predicted to impair their migratory efficiency. To test this idea, we examined, using a modified Boyden chamber (3-μm holes with collagen coating), whether fascin1 deficiency affects chemotaxis of mature DCs toward CCL19 (MIP3β). As Fig. 4A shows, fascin1 deficiency reduced chemotaxis by 42% (p = 0.0095).

Fascin1-deficient mice show reduced migration of Langerhans cells
Consistent with the impaired chemotaxis of fascin1 KO DCs toward CCL19 in vitro, we found that Langerhans cells of fascin1 KO mice showed reduced migration into lymph nodes. Twenty-four hours after painting of dorsal sides of both ears with an allergen of FITC, epidermal cell sheets were prepared and stained with a MHC-II Ab. Fig. 4B shows representative immunofluorescence...
images of wild and KO epidermal sheets. Without allergen, both wild-type and KO sheets showed a similar Langerhans cell distribution. After stimulation with the allergen, Langerhans cells from wild-type mice clearly exhibited decreased cell density when compared with KO. Quantitative data (Fig. 4C) indicate that the mean density of wild-type Langerhans cells after stimulation was about half of that of fascin1 KO (p < 0.001), whereas the density of Langerhans cells before stimulation was statistically similar between wild-type and fascin1 KO mice.

To confirm that the above difference in Langerhans cell density is indeed due to migration of Langerhans cells into lymph nodes, we measured the number of FITC-bearing DCs in draining lymph nodes following stimulation by FITC painting for 24 h. As Fig. 4D shows, there were over twice as many FITC-bearing DCs per lymph node in wild-type mice as in fascin1-null mice (p = 0.0059). Taken together, these results show that Langerhans cell migration is impaired in fascin1 KO mice, and support our hypothesis that fascin1 plays a critical role in DC migration into lymph nodes by promoting podosome disassembly and increasing dynamics of membrane protrusions.

**Fascin1-deficient DCs are more spread and thinner with fewer membrane protrusions**

Fascin1-deficient, mature DCs are morphologically very different from their wild-type counterparts. Fig. 5A shows scanning electron microscopy of wild-type and fascin1-null mature DCs. Fascin1-deficient DCs were much thinner and more spread with fewer and smaller dorsal ruffles than wild-type DCs. To quantitatively assess these shape changes, cells were labeled with rhodamine phalloidin and anti-CD86 Ab, and analyzed by immunofluorescence microscopy, using serial Z-section imaging (0.2 μm spacing) and three-dimensional rendering. Because the expression of CD86 correlates with that of fascin1 (Fig. 1Ae), CD86$^{\text{high}}$ DCs were chosen for morphological analysis. Fig. 5B shows representative images of wild-type and KO CD86$^{\text{high}}$ DCs stained with phalloidin. Orthogonal images in both xz and yz planes clearly showed that fascin1-deficient DCs were thinner than wild type. Statistical analyses using box plots (Fig. 5C) revealed a significant difference in thickness (p < 0.0001). The median thickness of fascin1-deficient DCs (n = 126) was 7.4 μm, whereas that of wild type (n = 196) was 10.7 μm. The difference in the thickness became even more prominent when DCs were centrifuged at 110 × g for 4 min; fascin1-deficient DCs were greatly flattened, with a median thickness of 3.6 μm (n = 55), whereas wild-type DCs were more resistant with a median thickness of 6.9 μm (n = 45). These results suggest that DC stiffness may be impaired in fascin1-deficient DCs.

To determine how fascin1 deficiency affects cell spreading, we made area measurements of xy images on the ventral surface. As the box plot of Fig. 5D shows, fascin1-deficient DCs are 40% more spread: the median area covered by fascin1-deficient DCs...
A-fascin1 (g–i) fascin1 mutants, and stained with anti-vinculin Ab (red; b background; pink bar) were also examined for podosome assembly. Dfascin1, GFP A-fascin, or GFP D-fascin, and cells were categorized as having less than four podosomes or more than five podosomes.

**FIGURE 2.** Fascin1 expression and actin-bundling activity are critical for podosome disassembly of mature DCs. A, Immunofluorescence of immature (a, b) and mature (c, d) DCs from wild-type (a, c) and fascin1-null (b, d) mice labeled with anti-vinculin (red) and anti-CD11c (green) Abs. Arrows, podosomes with the characteristic ring structure; arrowheads, focal adhesions. Scale bar, 15 μm. B, Mature fascin1high DCs (arrow) show no podosome assembly. Scale bar, 10 μm. C, Statistical analyses of podosome assembly. CD11c-positive immature (blue bar) and mature (red) DCs with at least five vinculin-positive podosomes were judged as DCs with podosomes. Wild-type, mature DCs with very high fascin1 expression (>10 times higher than background; pink bar) were also examined for podosome assembly. D, Effects of forced expression of GFP control (a–c), GFP wild-type fascin1 (d–f), GFP A-fascin1 (g–i), and GFP D-fascin1 (j–l) on podosome assembly. Fascin1-null DCs were transfected with GFP control (a–c), as well as with wild-type and fascin1 mutants, and stained with anti-vinculin Ab (red; b, e, h, k). GFP signal (green; a, d, g, j); merged images (c, f, i, l). Arrowheads in j–l show podosomes. Scale bar, 10 μm. E, Statistical analyses of podosome loss. Podosomes were counted in DCs exogenously expressing control GFP, GFP W-fascin1, GFP A-fascin, or GFP D-fascin, and cells were categorized as having less than four podosomes or more than five podosomes.

was 290 μm² (n = 95), whereas that of wild-type DCs was 205 μm² (n = 121), with a statistical significance (p = 0.0032). As wild-type DCs were 30% thicker than fascin1-deficient DCs, these measurements suggest that both types of DCs have roughly equal cell volumes.

The size and shape of protrusions varied widely, making simple measurements of the number and length of protrusions inappropriate for quantitative analyses. We thus measured circularity ([4π × [area]/[perimeter]²) of projected images generated from Z-section images, because more protrusions result in higher deviation from circularity (the value for a complete circle is 1). Box plot analyses (Fig. 5E) showed that fascin1 deficiency increased the median values of circularity from 0.39 (n = 112) to 0.51 (n = 120) with the statistical significance of p < 0.0001, confirming that fascin1 KO DCs have fewer protrusions. The finding that fascin1-null DCs show reduced numbers of protrusions is consistent with previous studies demonstrating the role of fascin1 in generating membrane protrusions in other cell types (16, 26, 29, 39, 40).

**Fascin1-deficient DCs fail to disassemble podosomes upon maturation**

Podosomes are disassembled in mature DCs (4, 10, 11). The images at the ventral surface of fascin1 KO DCs (Figs. 1Dh, 5Bb) showed many more podosome-like F-actin dots than in wild-type DCs, suggesting a difference in podosome dynamics. We found that this is the case. Fig. 2A shows immunofluorescence images of vinculin-labeled (red) immature and mature DCs from wild-type and fascin KO mice. In each case, DCs were identified by counterstaining with CD11c (green). Immature DCs from both wild-type and fascin1 KO DCs assembled podosomes to a similar extent. This result is consistent with the observation that fascin1 expression is minimal in immature DCs. As reported (4, 11), podosomes disappeared in most mature wild-type DCs. In sharp contrast, mature fascin1 KO DCs retained podosomes. We performed quantitative analyses of podosome number in CD11c-positive DCs by setting a criterion that DCs with a cluster of more than five podosomes (defined as vinculin-positive ring-like structures) were judged as podosome positive. Such measurements (Fig. 2C) revealed that, whereas the percentage of wild-type DCs with podosomes decreased from 65 (n = 117) to 22% (n = 166) upon maturation, the percentage of podosome-positive, fascin1-deficient DCs was unchanged by maturation (61% for immature, n = 158 and 59% for mature DCs, n = 111).

We found that the loss of podosomes is highly correlated with the extent of fascin1 expression. As shown in our FACS analyses (Fig. 1Ad, 1Ae), mature wild-type DCs can be grouped into two populations, one (∼40–50% of mature wild-type DCs) showing two orders of magnitude higher expression of fascin1 than the other. We examined the presence of podosomes in such fascin1high DCs by double staining with the anti-fascin1 and anti-vinculin Abs (Fig. 2B). We found that virtually all DCs expressing the higher level of fascin1 (n = 100) had no podosomes (pink bar, Fig. 2C), suggesting an important role for fascin1 in podosome loss. This
notion is consistent with the observation that the timing of fascin1 induction (∼7 h after LPS treatment) roughly corresponds to the time when mature DCs lose podosomes. The high correlation between high fascin1 expression and podosome loss may point to a specialized DC subset or maturation state with high fascin1 expression.

**Forced expression of fascin1 results in podosome loss**

The above correlation has prompted us to test whether very high expression of fascin1 in mature DCs is responsible for podosome loss. To this end, we forced expression of GFP fascin1 in fascin1-deficient DCs and counterstained them with the anti-vinculin Ab. As a control, GFP alone was transfected in a similar way. As Fig. 2Dd–f shows, the introduction of GFP fascin1 resulted in podosome loss in most DCs. In contrast, podosomes remained assembled in fascin1 KO DCs expressing control GFP (Fig. 2Da–c). Measurements of podosomes in transfected cells (Fig. 2E) revealed that most (76%) of DCs expressing GFP fascin1 (n = 113) exhibited four or fewer podosomes, whereas only 28% of DCs expressing control GFP (n = 45) displayed fewer than four podosomes. These results suggest that high levels of fascin1 in mature DCs are responsible for podosome loss in mature DCs.

**Actin bundling by fascin1 is critical for podosome loss**

We next examined whether podosome loss depends on the actin-bundling activity of fascin1. Actin-bundling activity of fascin1...
Fascin1 is critical for chemotaxis and Langerhans cell migration into draining lymph nodes. A, In vitro chemotaxis toward the chemokine CCL19, measured with a modified Boyden chamber (p = 0.0095). B, Immunofluorescence imaging of Langerhans cells in ear epidermal sheets from wild-type (a, c) and fascin1-null (b, d) mice without (control; a, b) and with FITC painting (+allergen; c, d). Langerhans cells (indicated by arrowheads) were labeled with the MHC-II Ab. Representative images from four independent experiments. C, Box plot analyses of Langerhans cell distribution without or with allergen treatment. NS, no statistical significance; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. D, Box plot analysis of FITC-bearing DCs migrated into draining lymph nodes. p = 0.0059.

Fascin1 is associated with the actin structure of podosomes

To explore how high fascin1 expression leads to podosome disassembly, we examined whether fascin1 binds to F-actin within podosomes. Because high expression of fascin1 in wild-type DCs makes it difficult to determine possible localization of fascin1 at podosomes in these cells, we searched for a hematopoietic cell line that expresses a low level of fascin1 and, at the same time, has podosome structures. We found that in contrast to primary macrophages, THP-1 cells (human monocytic leukemia cells) express a low level of fascin1, yet assemble podosomes when differentiated into macrophages by addition of phorbol ester (33). Double labeling of THP-1 cells with anti-fascin1 and anti-α-actinin Abs (Fig. 6A) clearly revealed colocalization of fascin1 (green) and α-actinin (red) at podosomes (arrowheads), with fascin1 being slightly inside the α-actinin ring structure.

The level of fascin1 in THP-1 cells is ~10 times lower than that of fascin1high mature DCs. We thus asked whether an increase in fascin1 concentration to the level observed with DCs could result in podosome disassembly in THP-1 cells. As Fig. 6B shows, micro-injection of GFP fascin1 induced podosome disassembly (Fig. 6Bc, 6Bd) in most (89%, 50 of 56 injected cells) THP-1 cells within 1 h (see Fig. 6C for quantitative data). Concomitantly, fascin1-injected cells frequently became rounded and detached from the substrate if incubated for a longer time. In contrast, most (66%, 38 of 58 injected cells) of control cells injected with FITC-labeled BSA retained podosomes (Fig. 6Ba, 6Bb), the percentage of which is statistically similar to that of un.injected cells (72%, see Fig. 6C). These results indicate that whereas fascin1 at a low concentration can bind to actin structure of the podosomes, high fascin1 expression as observed in DCs can cause podosome disassembly.

FIGURE 4. Fascin1 is critical for chemotaxis and Langerhans cell migration into draining lymph nodes. A, In vitro chemotaxis toward the chemokine CCL19, measured with a modified Boyden chamber (p = 0.0095). B, Immunofluorescence imaging of Langerhans cells in ear epidermal sheets from wild-type (a, c) and fascin1-null (b, d) mice without (control; a, b) and with FITC painting (+allergen; c, d). Langerhans cells (indicated by arrowheads) were labeled with the MHC-II Ab. Representative images from four independent experiments. C, Box plot analyses of Langerhans cell distribution without or with allergen treatment. NS, no statistical significance; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. D, Box plot analysis of FITC-bearing DCs migrated into draining lymph nodes. p = 0.0059.

is largely downregulated by phosphorylation at Ser39 (41, 42) because phosphorylation at Ser39 disrupts one of the two actin binding sites of fascin1. We thus expressed unphosphorylatable (A-fascin1, replacing Ser39 with Ala) and phosphomimetic (D-fascin1, replacing Ser39 with Asp) mutants in fascin1-null DCs. As shown in Fig. 2Dg–l, A-fascin1 was much more effective than D-fascin1 at inducing podosome loss. Indeed, quantitative data (Fig. 2E) revealed that A-fascin1 (n = 102) was slightly more effective in inducing podosome loss than wild-type fascin1 (W-fascin1), increasing the fraction of DCs without podosomes from 76 ± 8 to 85 ± 6% (p = 0.006). In contrast, D-fascin1 (n = 138) was much less effective than wild-type fascin1, resulting in only 42 ± 8% of the DCs displaying fewer than four podosomes (p < 0.0001). These results indicate that the actin-bundling activity of fascin1 is important for podosome disassembly, and suggest that phosphorylation of fascin1 could contribute to the regulation of podosome assembly.

Fascin1 is associated with the actin structure of podosomes

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FIGURE 5. Morphological characterization of wild-type and fascin1-null DCs. A, Scanning electron micrographs of wild-type (a) and fascin1-deficient (b) mature DCs. Scale bars, 10 μm. B, Orthogonal views of wild-type (a) and fascin1-deficient (b) CD86high DCs. Mature DCs were double stained with phalloidin and anti-CD86 Ab. Only phalloidin staining is shown here. The xy images are on the ventral surface. Both xy and yz images are shown with the top and bottom of cells indicated by dashed lines. Scale bars, 5 μm. C–E, Box plot analyses of thickness (C, area (D), and circularity (E) of wild-type and fascin1-deficient DCs. Thickness was determined without (w/o cfg) or with (w. cfg) cytospin.
each condition were counted for each set of experiments. Dependent experiments are shown. Approximately 20 injected cells for fascin1 (red) or FITC BSA (blue). Representative data from three in-podosome arrays of uninjected cells (green), or after microinjection of C. Arrows indicate podosomes. Whereas arrowheads indicate podosomes.

b: Cells were microinjected with FITC-labeled BSA (green) and anti-α-actinin at podosomes. THP-1 cells differentiated with 2-O-tetradecanoylphorbol-13-acetate were labeled with anti-fascin1 (b, e; green) and anti-α-actinin (red; a, d). Abs. c and f. Merged images. Images in d–f show enlargements of the boxed areas in a–c. Arrowheads, podosomes. B, Disassembly of podosomes by microinjection of fascin1. THP-1 cells were microinjected with FITC-labeled BSA (a) or GFP fascin1 (c) and counterstained with phalloidin (b, d). Arrows indicate injected cells, whereas arrowheads indicate podosomes. C, Percentage of cells with podosome arrays of un.injected cells (green), or after microinjection of fascin1 (red) or FITC BSA (blue). Representative data from three independent experiments are shown. Approximately 20 injected cells for each condition were counted for each set of experiments.

Discussion

We have demonstrated that fascin1 plays a critical role in the alterations in motility, morphology, and adhesion associated with DC maturation. Fascin1-null DCs, when fully matured, are more spread, show fewer and less dynamic membrane protrusions, and retain podosomes. Importantly, fascin1-null DCs show reduced directed migration both in vitro and in vivo.

Fascin1 is critical for dynamic dorsal ruffling

How does fascin1 enhance the dynamics of membrane protrusions? We found that fascin1 is colocalized with F-actin at the cortex of veil-like protrusions (Fig. 1D). Judging from its actin bundling and cross-linking activity, fascin1 is likely to form a meshwork of actin filaments at the cortex, which would give the cell cortical rigidity. Recent studies have shown that the actin cross-linking activity of fascin1 is extremely dynamic (29, 43, 44), and suggested that this dynamic cross-linking is required for vigorous movements of filopodia while at the same time maintaining sufficient rigidity for filopodial protrusions (43). A similar mechanism is likely to work for veil-like protrusions of mature DCs. As the DC cortex protrudes and retracts, fascin1 would be able to quickly reorganize the actin meshwork, providing both the rigidity and the flexibility needed to support dynamic membrane protrusions. Such dynamics are likely to be critical for DC migration through tissues and extracellular matrix to reach the lymph nodes. In keeping with this idea, we found that Langerhans cells from fascin1 KO mice show reduced emigration into draining lymph nodes (Fig. 4B–D).

High fascin1 expression is likely to cause the disassembly of podosomes in mature DCs

We found that fascin1 expression is closely correlated with the loss of podosomes in mature DCs. Importantly, forced expression of fascin1 in fascin1-null DCs resulted in podosome disassembly (Fig. 2). Two recent studies, however, have shown that fascin1 appears to favor assembly of podosomes, as well as invadopodia, structures closely related to podosomes (45, 46). In platelet-derived growth factor-treated smooth muscle cells, fascin1 depletion has been reported to suppress podosome assembly (45). Likewise, fascin1 has been shown to stabilize F-actin in invadopodia in melanoma cells (46). We speculate that these apparently contradictory functions of fascin1 may be explained by the difference in fascin1 expression levels between mature DCs and other cell types. Mature DCs express fascin1 one order in magnitude higher than do other cells. In support of this notion, we demonstrated that podosome assembly could be controlled by altering fascin1 levels in THP-1 cells: whereas endogenous fascin1 is present at a low level in THP-1 cells and localized to podosomes, microinjection of a large amount of fascin1 caused disassembly of podosomes (Fig. 6B, 6C).

An important question is how high levels of fascin1 could cause podosome disassembly in mature DCs. Fascin1 at a low level binds to actin structure of podosomes (Fig. 6A), indicating that fascin1 and other actin-binding proteins can simultaneously bind to actin filaments. However, very high levels of fascin1 would saturate actin filaments [fascin1 can bind to actin at a molar ratio of 1 fascin1 to 4 actin molecules (47)], which would compete with other proteins for actin binding (48). Such competition would result in dissociation of an actin-binding protein(s) that is critical for the organization of podosomes, leading to disassembly of podosomes. Indeed, Park et al. (49) have shown that fascin1 debranches Arp2/3 complex-mediated branched filaments, transforming the dendritic filament assembly into actin bundles in vitro. Because Arp2/3 complex is an essential component of podosomes (50–53), debranching of Arp2/3-mediated dendritic filaments could block de novo synthesis and/or maintenance of podosomes. This idea is consistent with the result that A-fascin1 is much more effective in podosome disassembly than is D-fascin1 because D-fascin1 shows much weaker actin-bundling activity (Fig. 2).

Loss of podosomes may be critical for migration of mature DCs

Podosomes appear to profoundly alter migration patterns of DCs, at least in vitro. It has been reported that mature DCs without podosomes display high-speed migration with low adhesion to the substrate when compared with immature DCs with podosomes (10). Such high-speed migration with reduced adhesion would be advantageous for mature DCs to travel to a lymph node as quickly as possible for efficient presentation of Ags to naive T cells. In contrast, immature DCs need to move around the peripheral tissues to constantly sample foreign and host Ags. Such movement may require an adhesion structure like podosomes for attachment to the extracellular matrix (10, 13, 14, 54, 55). It is worth noting that other primary hematopoietic cells like macrophages have prominent podosomes, whereas no fascin1 expression was detected. These cells may need podosomes as adhesion structures so that they can move around the peripheral tissues as a sentinel against external pathogens.

The loss of podosomes might also be critical for the assembly of an immunological synapse. Geyeregger et al. (56) have shown that agonists of liver X receptors (LXRs) blocked fascin1 expression in human DCs and, at the same time, inhibited the assembly of the immunological synapse. Importantly, overexpression of fascin1 in LXR agonist-treated DCs restored immunological synapse assembly (56). We have found that LXR agonists block
podosome disassembly in mature, wild-type DCs (S. Yamashiro, unpublished results), again supporting our notion that the loss of fascin is highly correlated with sustained podosome assembly. Perhaps the disassembly of podosomes may facilitate the assembly of an immunological synapse because these two structures share molecular constituents (57).

In conclusion, we have demonstrated that fascin1 plays a critical role in chemotactic migration of DCs. Manipulation of fascin1 expression may thus be effective in enhancing DC-based immune therapy. For example, whereas tumor Ag-loaded DCs have been used as cancer vaccines, only a tiny fraction (1%) of DCs s.c. injected is able to migrate into lymph nodes of cancer patients (58). It might be possible to increase the efficiency of DC migration by selecting DCs with high fascin1 expression and/or by exogenously increasing fascin1 expression.

Acknowledgments

We thank Drs. Frank Deis and Barth Grant for critical reading of the manuscript. Drs. M. Mooseker and M. Krendel for help with DC preparations, and V. Starovoytov for help with scanning electron microscopy.

Disclosures

The authors have no financial conflicts of interest.

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