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Fascin Confers Resistance to *Listeria* Infection in Dendritic Cells

Fumio Matsumura,* Yoshihiko Yamakita,*1 Val Starovoytov,† and Shigeko Yamashiro*

Ag-presenting dendritic cells (DCs) must survive bacterial infection to present Ag information to naive T cells. The greater ability of DCs' host defense is evident from the report that DCs are more resistant to *Listeria monocytogenes* than macrophages. However, the molecular mechanism of this resistance is unclear. We found that *Listeria* replicate more slowly in wild-type DCs compared with fascin1 knockout DCs. This finding is significant because fascin1, an actin-bundling protein, is specifically and greatly induced upon maturation of dendritic cells, but not other blood cells, including macrophages and neutrophils. Infection by *Listeria* makes phagosomes more acidic in wild-type DCs than in fascin1 knockout DCs, suggesting that fascin1 facilitates phagolysosomal fusion for killing of phagocytosed *Listeria*. We further found that fascin1 binds to LC3, an autophagosome marker, both in vivo and in vitro. *Listeria* are associated with LC3 to a greater extent in wild-type DCs than in fascin1 knockout DCs, suggesting that fascin1 facilitates autophagy for eradication of cytoplasmic *Listeria*. Taken together, our results suggest that fascin1 plays critical roles in the survival of DCs during *Listeria* infection, allowing DCs to function in innate and adaptive immunity. The Journal of Immunology, 2013, 191: 6156–6164.

DCs have demonstrated to play a crucial role in eradication of *L. monocytogenes* in mice: DCs, but not macrophages, are essential for priming naive CTLs that are specific for *L. monocytogenes* Ags (8). In vitro, *L. monocytogenes* infection of bone marrow–derived DCs (BM-DCs) enhances their maturation, as well as their ability to stimulate T cell differentiation (9, 10). Importantly, BM-DCs have been shown to be more resistant to *L. monocytogenes* than are macrophages (11, 12). This resistance must be critical for DCs’ primary function of Ag presentation because DCs have to survive infection and present Ag information to naive T cells. Whereas limited escape of *L. monocytogenes* from phagosomes into the cytosol has been suggested to be a reason for the resistance (11, 12), the molecular mechanism(s) and molecule(s) for the higher killing activity shown by DCs are not clear. Such molecule(s) must be either specifically expressed or activated in DCs, but not in macrophages.

Fascin1 is a unique actin-bundling protein that is very highly and specifically induced upon maturation of DCs, but not expressed in other blood cells, including macrophages, neutrophils, T cells, and B cells (13). By characterizing DCs from fascin1 knockout (KO) mice, we have demonstrated that fascin1 causes large changes in the organization of the peripheral actin cytoskeleton of DCs: fascin1 makes veil-like dorsal membrane ruffling more vigorous and promotes in vitro chemotactic motility, as well as in vivo migration into draining lymph nodes (14). Consistent with the above result, genome-wide, expression profile analyses of mouse DCs isolated in vivo have revealed that fascin1 is abundantly expressed, in particular, in migratory DCs (15).

The fascin1-mediated, massive changes in the peripheral actin cytoskeleton could affect bactericidal activity of DCs because the actin cytoskeleton is involved in several aspects of host defense mechanisms: for example, bacterial entry via phagocytosis is controlled by the peripheral actin cytoskeleton (16–18). Assembly and bundling of actin filaments have been reported to regulate phagolysosomal fusion (19, 20). Autophagy, which is able to kill cytoplasmic bacteria that have escaped from phagosomes, requires actin and actin-binding proteins, including Arp2/3 and cortactin (21–23). Fascin1 might increase cell-to-cell transmission of *L. monocytogenes* from phagosomes into the cytosol has been suggested to be a reason for the resistance (11, 12), the molecular mechanism(s) and molecule(s) for the higher killing activity shown by DCs are not clear. Such molecule(s) must be either specifically expressed or activated in DCs, but not in macrophages.

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Abbreviations used in this article: BM-DC, bone marrow–derived dendritic cell; CMFDA, 5-chloromethyl fluorescein diacetate; DC, dendritic cell; KO, knockout; LLO, listeriolysin O; moi, multiplicity of infection; PLA, proximity ligation assay; SLAP, spacious *Listeria*-associated phagosome.

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DCs expressing high levels of fascin1 are free from L. monocytogenes infection, suggesting critical roles of fascin1 in bacterial eradication.

Materials and Methods

Abs and reagents

The following Abs were used: FITC-conjugated hamster anti-mouse CD11c mAb, FITC-conjugated rat anti-mouse CD68 (B7-2) mAb, FITC-conjugated rat anti-mouse I-A/E (MHC-II) mAb, rabbit anti-Ln polyclonal Ab (BD Biosciences, San Jose, CA), rabbit anti-LC3 polyclonal Ab, mouse anti-LC3 mAb (MBL international, Woburn, MA), mouse anti-fascin mAb (55k-2), rabbit anti-fascin mAb, and rabbit anti-actin mAb (Cell Signaling Technology, Danvers, MA). GM-CSF was purchased from Invirogen (Carlsbad, CA). A recombinant fusion, fascin1 protein, was prepared, as described previously (26). A recombinant GST-LC3 fusion protein was expressed in bacteria, as described (27).

Preparation of BM-DCs

Fascin1 KO heterozygous mice were generated by Lexicon Pharmaceuticals (Woodlands, TX) from an ES cell line (OST124903) (Lexicon’s OmniBank gene library of gene KO ES cell clones) and backcrossed with C57BL/6 female mice for >14 generations (28). DCs were prepared from bone marrow isolated from fascin1 KO homozygous mice and their wild-type littermates, as described previously (14). 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.

Infection of DCs with L. monocytogenes infection and measurements of CFUs

Infection of DCs with L. monocytogenes was performed essentially as described in Westcott et al. (11, 12). Briefly, DCs were infected with a wild-type strain of L. monocytogenes (10403S, a gift of D. A. Portnoy, University of California at Berkeley) at a multiplicity of infection (moi) of 0.5, except specified otherwise. At 30 min postinfection, DCs were gently washed with fresh culture media (DMEM containing 10 ng/ml GM-CSF), and gentamicin (final, 10 μg/ml) was added to kill extracellular bacteria (gentamicin is present throughout the entire experiments). At varying times postinfection, DCs were harvested and washed three times with PBS, and 10% of cells was used for counting viable cell numbers with a hemocytometer, following staining with trypan blue. The rest of DCs were homogenized in PBS containing 0.1% Triton X-100. Cell disruption was confirmed by phase-contrast microscopy. The homogenates were serially diluted and plated on Luria-Bertani agar plates overnight at 37˚C to determine CFU.

Measurements of bacterial uptake

To determine whether fascin1 affects bacterial uptake, wild-type and fascin1 KO DCs were infected with L. monocytogenes at a moi of 20. At 30 min postinfection, gentamicin (10 μg/ml) was added to kill extracellular bacteria. DCs were washed three times with PBS and homogenized in PBS with 0.1% Triton X-100. Bacteria incorporated into DCs were measured by determining CFU, as described above.

Immunofluorescence of L. monocytogenes-infected DCs

Infection with L. monocytogenes caused rapid maturation of DCs, as judged from the expression of fascin1, CD86, and MHC-II: fascin1 expression was clearly observed as early as 60 min after L. monocytogenes infection. This is in contrast to DC maturation by LPS, which takes as long as 7 h after the addition of LPS (14).

L. monocytogenes infection changed the morphology of DCs to a more rounded form that tended to detach from the substrate. To keep rounded DCs on coverslips for immunofluorescence staining, DCs were cytospun (500 × g for 5 min), fixed with methanol, and processed for immunofluorescence, as described previously (14). In some cases, DCs were fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and processed for immunofluorescence, as described (14). Images were taken as Z-stacks (0.2 μm spacing) with a DeltaVision Image Restoration Microscope system (Applied Precision Instrument, Issaquah, WA), deconvolved either with the softWoRx software (Applied Precision Instruments) or the Huygens software (Applied Precision Instrument, Issaquah, WA), and ratio images of a FITC fluorescence intensity divided by a corresponding rhodamine fluorescence intensity was plotted against the pH of each buffer.

For a vacuolar pH measurement, a LysoSensor yellow/blue dye (DN-160; Molecular Probes) was used. This dye is incorporated into acidic organelles, including phagosomes and lysosomes, and ratio imaging of 525 nm/470 nm gives pH values. DCs were labeled with 20 μM LysoSensor dye for 2 min and washed with DMEM containing 10% FCS. Images were taken with DeltaVision microscopy with an excitation wavelength of 360 ± 40 nm and two emission wavelengths of 457 ± 30 nm and 528 ± 38 nm, and ratio images of 528 nm emission divided by 457 nm emission were generated with the SoftWoRx software. Live-cell ratiometric imaging was made before infection and 30 min, 1 h, and 3 h postinfection (moi of 3). To convert the ratio to pH, LysoSensor-labeled DCs were permeabilized with 0.1% saponin, fixed with 3.7% formaldehyde at varying pH (3–7.5) containing 0.1% Triton X-100 or 1 μM nigericin and valinomycin (Sigma-Aldrich), and the ratios were determined as described above.

Autophagy analyses

At varying times postinfection, DCs were cytospun and fixed with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and permeabilized with an anti-LC3 and anti-L. monocytogenes Ab. DCs were imaged using a DeltaVision microscope system, as described above, and L. monocytogenes-associated LC3 were quantitated and expressed as percentages of association.

Fascin1–LC3 binding

Fascin1–LC3 interaction was examined by coimmunoprecipitation and a GST pull-down assay. For immunoprecipitation, HELa cells were homogenized in an immunoprecipitation buffer containing 50 mM Tris-HCl, 50 mM NaCl, 0.05% Triton X-100, 0.2 mM EDTA, 0.1 mM EGTA, 5 mM MgCl2, and 10% glycerol (pH 7.5), and the homogenates were centrifuged at 16,000 × g for 10 min without a break. Anti-LC3 Ab was added to the supernatants. An anti-LAMP1 Ab was used as a control. The immune complexes were collected by addition of protein A Sepharose beads (GE Healthcare, Piscataway, NJ), separated on SDS-PAGE, and immunoblotted with anti-LC3, anti-LAMP1, and anti-fascin1 Abs.

A GST pull-down assay was performed, as described previously (30). Briefly, GST-LC3 that had been bound to glutathione–Sepharose 4B beads (GE Healthcare) was incubated with GFP-fascin1 (final 0.5 mg/ml) for 45 min in the immunoprecipitation buffer described above. After centrifugation at 600 × g for 5 min, glutathione beads were washed extensively with the immunoprecipitation buffer, lysed in SDS-PAGE sample buffer, and analyzed by Western blotting to detect binding between LC3 and GFP-fascin1. As a control, GST alone was bound to glutathione–Sepharose beads and incubated with GFP-fascin1 in the same way as above. The bead-bound fraction was processed in the same way as above.

Proximity ligation assay

Proximity ligation assay (PLA) was performed according to the manufacturer’s instruction (O-link Bioscience, Uppsala, Sweden). Briefly, DCs were fixed with methanol at −20˚C for 30 s, then further fixed with 3.7% formaldehyde for 5 min, and permeabilized with 0.2% Triton X-100. This

Analyses of phagosomal and vacuolar pH

A mutant strain L. monocytogenes (DP L-2319, Δhly ΔploA ΔploB, a gift of D. A. Portnoy, University of California at Berkeley) was used for measurements of pH of L. monocytogenes-engulfed phagosomes. As this strain lacks LLO and two phospholipases, it is defective in vacuolar lysis, and phagosomal pH was determined by ratiometric imaging of phagosomal L. monocytogenes essentially as described (12). Briefly, exponentially growing L. monocytogenes were washed three times with PBS and then labeled with CMFDA (100 μM) for 10 min at 37˚C. The labeling reaction was stopped by addition of PBS containing 10% FCS. CMFDA-labeled L. monocytogenes were washed after three times and labeled with 0.2 mg/ml tetramethylrhodamine at 37˚C for 45 min. The reaction was stopped with PBS containing 10% FCS.

One hour postinfection with double-labeled L. monocytogenes at a moi of 3, DCs were imaged in the FITC and rhodamine channels with a DeltaVision microscopy system, and ratio images of FITC to rhodamine were generated with the softWoRx software (Applied Precision Instruments). For conversion from the ratio to the pH, double-labeled L. monocytogenes were incubated in PBS at varying pH from 3 to 7.5 (adjusted with HCl), and the ratio of a FITC fluorescence intensity divided by a corresponding rhodamine fluorescence intensity was plotted against the pH of each buffer.

For a vacuolar pH measurement, a LysoSensor yellow/blue dye (DN-160; Molecular Probes) was used. This dye is incorporated into acidic organelles, including phagosomes and lysosomes, and ratio imaging of 525 nm/470 nm gives pH values. DCs were labeled with 20 μM LysoSensor dye for 2 min and washed with DMEM containing 10% FCS. Images were taken with DeltaVision microscopy with an excitation wavelength of 360 ± 40 nm and two emission wavelengths of 457 ± 30 nm and 528 ± 38 nm, and ratio images of 528 nm emission divided by 457 nm emission were generated with the SoftWoRx software. Live-cell ratiometric imaging was made before infection and 30 min, 1 h, and 3 h postinfection (moi of 3). To convert the ratio to pH, LysoSensor-labeled DCs were permeabilized with 0.1% saponin, fixed with 3.7% formaldehyde at varying pH (3–7.5) containing 0.1% Triton X-100 or 1 μM nigericin and valinomycin (Sigma-Aldrich), and the ratios were determined as described above.

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fixation method that was previously used to simultaneously visualize fascin1 and actin (25) was found to be suitable for simultaneous visualization of fascin1 and LC3. Fixed DCs were incubated with anti-fascin1 mouse mAbs and anti-LC3 rabbit polyclonal Abs. The mouse and rabbit Abs were labeled with anti-mouse and anti-rabbit PLA probes, each of which was conjugated with oligonucleotides. If these two oligonucleotides were located at a close proximity (within ~40 nm), they were ligated to form a closed circle, which was amplified with DNA polymerase and detected with fluorescently labeled oligonucleotides. As a positive control, DCs were labeled with anti-fascin1 and anti-actin Abs, the Ags of which are known to interact with each other (25, 31).

Electron microscopy

Thin-section electron microscopy was performed according to protocols by the electron microscopy facility at Rutgers University. Briefly, DCs were infected with wild-type strain *L. monocytogenes* at a moi of 1, and gentamicin was added at 30 min postinfection. At 50 min postinfection, DCs were fixed with 2% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer for 5 min, scrape-harvested into an Eppendorf tube, further fixed in the same solution for 55 min, washed with PBS, and postfixed with 1% OsO₄ for 1 h. After washing with PBS, samples were dehydrated with graded solutions of ethanol and cells were embedded with a low viscosity embedding media kit (Electron Microscopy Sciences, Hartford, PA). Thin sections were observed with a JEOL 100CX microscope (Peabody, MA) at 80 kV, and ~200 bacteria were imaged for each experiment.

Statistical analysis

Statistical analyses were performed using unpaired, two-tailed, Student *t* test (http://www.physics.csbsju.edu/stats/t-test.html).

Results

Fascin1 KO DCs are more susceptible to *L. monocytogenes* infection than wild-type DCs

We examined, by infecting DCs at a moi of 0.5, whether fascin1 affects replication rates of intracellular bacteria in wild and fascin1 KO DCs. At this moi, both wild and fascin1 KO DCs were mostly viable during the initial 6-h incubation, allowing us to determine bacterial replication rates. Measurements of intracellular bacterial numbers at 1, 2, 4, and 6 h (Fig. 1A) revealed that *L. monocytogenes* replicated faster in fascin1 KO DCs than in wild-type DCs. During the initial 2 h, *L. monocytogenes* grew in a similar rate in both wild-type and fascin1 KO DCs. However, *L. monocytogenes* growth became much slower at 4 and 6 h in wild-type DCs, whereas *L. monocytogenes* grew more consistently in KO DCs. The doubling times (estimated from CFU between 1 and 4 h) were 45 min in fascin1 KO DCs and 90 min in wild-type DCs. The longer doubling time of *L. monocytogenes* in wild-type DCs, as well as the growth inhibition at ~4 h, is consistent with the previous report for wild-type DCs by Westcott et al. (11). The susceptibility to *L. monocytogenes* infection in fascin1-deficient DCs is further confirmed by tracking viability of DCs for longer incubation. As Fig. 1B shows, cell number for fascin1 null DCs decreased much more rapidly at 24 and 48 h than that of wild-type DCs. At 24 h postinfection the bacterial number (9.4 × 10⁵) of fascin1 KO DCs was twice as high as that (4.6 × 10⁶) of wild-type DCs. However, this difference in bacterial number is an underestimate because the cell number of KO DCs was twice as low as that of wild-type DCs (Fig. 1B).

The bacterial numbers at 1 h postinfection were similar between wild-type and fascin1 KO DCs, suggesting that the resistance of wild-type DCs to *L. monocytogenes* infection is not due to a difference in phagocytic activity. To confirm this, we measured bacterial uptake at 30 min postinfection. To increase an accuracy of measurements of bacterial uptakes, DCs were infected at a moi of 20. As Fig. 1C shows, both wild-type and fascin1 KO DCs ingested similar number of bacteria during 25 min of phagocytosis. Student *t* test revealed no statistical significance (*p* = 0.57).

**FIGURE 1.** Higher susceptibility of fascin1 KO DCs to *L. monocytogenes* infection. (A) *L. monocytogenes* replication during the initial 6 h of infection. Wild-type (wild) and fascin1 KO (KO) DCs were infected with *L. monocytogenes* at a moi of 0.5 for 30 min. After killing extracellular *L. monocytogenes* with gentamicin, growth of intracellular *L. monocytogenes* was measured, as described in Materials and Methods. (B) Viability of wild-type (wild) and fascin1 KO (KO) DCs for 48 h postinfection. More wild-type DCs can survive than fascin1 KO DCs. (C) Bacterial uptake after 30 min postinfection. No statistically significant difference in phagocytic activities between wild-type and fascin1 KO DCs was observed. Representative results are shown from at least three independent experiments. Error bars, SD.

DCs with fascin1 expression are resistant to *L. monocytogenes* infection

Immunofluorescence revealed that wild-type DCs with high fascin1 expression are very resistant to *L. monocytogenes* infection (Fig. 2). DCs were infected with *L. monocytogenes* at a moi of 3, and, at 3 and 24 h postinfection with L. monocytogenes, wild-type (Fig. 2A, 2C), and fascin1 KO (Fig. 2B, 2D) DCs were stained with an anti-*L. monocytogenes* Ab and counterstained with an anti-fascin1 (for wild-type DCs) or anti-CD86 Ab (for KO DCs). At 3 h postinfection, wild-type DCs with low levels of fascin1 expression (labeled green) were infected with several *L. monocytogenes*
FIGURE 2. Fascin1-expressing DCs are resistant to L. monocytogenes infection. (A–D) Immunofluorescence images of wild-type (A, C) and fascin1 KO (B, D) DCs infected with L. monocytogenes for 3 h (A, B) and 24 h (C, D). DCs were infected with L. monocytogenes at a moi of 3.0. At indicated time, wild-type DCs were stained with anti-L. monocytogenes (red) and anti-fascin1 (green) Abs, and fascin1 KO DCs were stained with anti-L. monocytogenes (red) and anti-CD86 (green) Abs. Scale bar, 10 μm. (E) Quantitative analyses of L. monocytogenes infection in fascin1-positive, wild-type DC (red bars); fascin1-negative, wild-type DCs (green); and fascin1 KO DCs (blue). Twenty-four hours postinfection, wild-type, fascin1-positive DCs (n = 49), as well as wild-type, fascin1-negative DCs (n = 94), and fascin1 KO (n = 100) DCs, were analyzed by immunofluorescence, and the number of intracellular L. monocytogenes per DC was counted. For each type of DC, noninfected cells and cells infected with one, two to four, or more than five L. monocytogenes were expressed as percentages of total cells. The p values of L. monocytogenes infection between fascin1-positive, wild-type DCs, and fascin1-negative wild-type DCs and between fascin1-positive, wild-type DCs, and KO DCs are <0.0001. In contrast, the difference between fascin1-negative, wild-type DCs, and fascin1 KO DCs is statistically insignificant. Error bars, SD. Representative results from three independent experiments.

(arrows in Fig. 2A; L. monocytogenes labeled red). At 24 h postinfection (Fig. 2C), some DCs expressed high levels of fascin1 (indicated by asterisks). Remarkably, these DCs with high fascin1 expression had no L. monocytogenes infection, whereas adjacent fascin1-negative DCs (indicated by an arrowhead) were heavily infected. The presence of fascin1-positive and fascin1-negative DCs in wild-type DC preparations is consistent with our previous report that LPS-matured, CD11c-positive DCs from wild-type mice have two distinct populations in terms of fascin1 expression. One population (~40% of total DCs) shows two orders of magnitude higher fascin1 expression than the other (14).

Fascin1 KO DCs infected with L. monocytogenes at 3 h postinfection started to express CD86 (Fig. 2B), a maturation marker, indicating that KO DCs also became matured by L. monocytogenes infection. Unlike wild-type DCs, however, fascin1 KO DCs were heavily and uniformly infected with L. monocytogenes at 24 h (Fig. 2D). Quantitative analyses (Fig. 2E) confirmed the above observations: no L. monocytogenes was detected in 90% of DCs with high fascin1 expression at 24 h postinfection (red bars). In contrast, >80% of fascin1 KO DCs were heavily infected (blue bars). Fascin1-negative, wild-type DCs (green bars) were infected with L. monocytogenes as heavily as fascin1 null DCs, suggesting that fascin1 expression is well correlated with the resistance to L. monocytogenes infection in DCs.

Wild-type DCs exhibit phagosomal acidification to a higher extent than fascin1 KO DCs

DCs have to control proliferation of L. monocytogenes in two major steps. The first one is to eradicate phagosome-engulfed L. monocytogenes, and the second is to kill cytoplasmic L. monocytogenes that have escaped from phagosomes. Phagolysosomal fusion is one of the primary host defense systems that can kill phagosome-entrapped bacteria. Because phagolysosomal fusion is controlled by the actin cytoskeleton (19, 20), fascin1 may alter this fusion process. Because the fusion efficiency depends on the phagosomal pH (32), we measured phagosomal pH. We found that wild-type DCs showed lower phagosomal pH than fascin1 KO DCs (Fig. 3). To determine phagosomal pH, we used fluorescently labeled L. monocytogenes as a pH indicator (L. monocytogenes double labeled with pH-sensitive [CMFDA] and pH-insensitive [rhodamine] dyes) and performed ratiometric imaging. This is based on the observation that CMFDA fluorescence is lower in an acidic environment, whereas rhodamine fluorescence is independent of pH. A L. monocytogenes mutant (DP L-2319) that is unable to escape from phagosomes was used for infection to confirm that L. monocytogenes is kept contained within phagosomes during the experimental time window. Fig. 3A shows representative images of wild-type (Fig. 3Aa–c) and fascin1
Figure 3. Phagosomal pH is lower in wild-type DCs than in fascin1 KO DCs at 1 h postinfection. (A) Fluorescent images at 529 nm (CFMDA), 617 nm (rhodamine), and corresponding ratiometric (529 nm/617 nm) images of CFMDA/rhodamine–double-labeled L. monocytogenes phagocyted in DCs. A L. monocytogenes mutant DP L-2319 (ΔplyA ΔplcB) was used for infection (moi of 3) to keep L. monocytogenes entrapped in phagosomes. Ratio (529 nm/617 nm) images revealed that phagosomal pH in wild-type DCs is lower than that in fascin1 KO DCs. Arrowheads, phagocytosed L. monocytogenes. Cell peripheries are indicated by dashed lines. (B) Box plot of phagosomal pH determined by ratiometric analyses of CFMDA/rhodamine–double-labeled L. monocytogenes, as described in Materials and Methods. Representative results are shown from three independent experiments.

KO (Fig. 3Ad–f) DCs at 1.5 h postinfection with fluorescently labeled L. monocytogenes (Fig. 3Aa, 3Ad, 529 nm; Fig. 3Ab, 3Af, 617 nm; Fig. 3Ac, 3Af, ratio image). Ratio images (Fig. 3Ac, 3Af) indicate that L. monocytogenes in wild-type DCs show lower ratios than L. monocytogenes in fascin1 KO DCs. This observation indicates that phagosomes of wild-type DCs are in a more acidic environment. Quantitative ratiometric analyses with box plots confirmed this (Fig. 3B): the median phagosomal pH was 5.6 (n = 44) for wild-type DCs and 6.0 (n = 87) for fascin1 KO DCs (p < 0.001). The pH value (pH 5.6) of wild-type DCs is similar to what was recently reported by Westcott et al. (12).

To expand the above analyses, we used a LysoSensor yellow/blue dye (DN-160; Molecular Probes) and examined whether fascin1 affects the pH of intracellular acidic vacuoles. This dye is incorporated into acidic organelles, including phagosomes, late endosomes, and lysosomes; and ratio imaging of 528 nm/457 nm gives pH values. After incorporation of the dye, DCs were infected with wild-type L. monocytogenes, and ratiometric imaging was performed at different time points. Fig. 4A shows representative images at 457 nm and 528 nm emission, as well as their corresponding ratiometric images. Before infection (Fig. 4Aa–f), the ratiometric images of both wild-type (Fig. 4Aa–f) and fascin1 KO (Fig. 4Ad–f) DCs were dark, indicating that vacuolar pH was neutral for both wild-type and fascin1 KO DCs. After L. monocytogenes infection (Fig. 4Ag–m), however, ratiometric images showed many brighter vacuoles in wild-type DCs (Fig. 4Ag–i) than in fascin1 KO DCs (Fig. 4Ak–n). In addition, infected L. monocytogenes was also labeled with this dye (indicated by arrowheads) and exhibited brighter ratiometric values in wild-type DCs than in fascin1 KO DCs.

Fig. 4B shows the time course of vacuolar pH change determined by the above ratiometric analyses. Prior to infection, the vacuolar pH was neutral to alkaline (7.0–7.2) in both wild-type and fascin1 KO DCs. L. monocytogenes infection induced rapid acidification of the vacuolar pH. Importantly, wild-type DCs exhibited a greater extent of acidification than did fascin1 KO DCs. At 1–3 h postinfection, the vacuolar pH of wild-type DCs became acidic to the range between 4.6 and 5.0. In contrast, the vacuolar pH of fascin1 KO DCs was lowered to a lesser extent, ranging between 5.4 and 5.8 during the same time window (p < 0.0001). These results suggest that fascin1 is critical for acidification of not only phagosomes, but also other acidic vacuoles, including late endosomes, phagolysosomes, and lysosomes.

Fascin1 binds to LC3, a component of autophagosomes

Autophagy is known to eradicate L. monocytogenes that have escaped into the cytoplasm from phagosomes. Because autophagy is controlled by the autophagy process. Inspection of the fascin1 sequence revealed that fascin1 contains two conserved sequences of Y152AHL155 and Y314WTL317 (mouse fascin1 sequence, NP_032010), which corresponds to a motif for the LC3-interacting region (W/F/Y-X-X-L/I/V) (34, 35). Indeed, we found that fascin1 binds to LC3 in vivo, as well as in vitro. As Fig. 5A shows, fascin1 was detected in LC3 immunoprecipitates from HeLa cells (Fig. 5A, lane 2), suggesting that LC3 and fascin1 form a complex in vivo. As a control, we examined whether fascin1 is present in LAMP1 immunoprecipitation and found that fascin1 was not detected in LAMP1 immunoprecipitates (Fig. 5A, lane 1). The association between LC3 and fascin1 appears to be direct, as measured with in vitro binding assays. GST pull-down assays (Fig. 5B) showed that GST-LC3 bound to fascin1 (Fig. 5B, lane 3), but not GST alone (Fig. 5B, lane 2).
A PLA revealed that both fascin1 and LC3 in wild-type DCs were colocalized as a complex inside DCs. In a PLA system, two proteins are probed with Abs conjugated with short nucleotide oligonucleotides. As Fig. 5C shows, speckle-like fluorescent spots were detected in the perinuclear region of L. monocytogenes-infected, wild-type DCs (Fig. 5Ca–c). In contrast, no fluorescent signals were detected in fascin1 KO DCs (Fig. 5Cd–f), confirming that the fluorescent signals in wild-type DCs indeed represent complex formation between fascin1 and LC3. To validate the authenticity of the PLA, we examined whether this assay can detect the known association between fascin1 and actin (31). Unlike the perinuclear localization of fascin1–LC3 complexes, a PLA assay to detect fascin1–actin association revealed many speckle-like fluorescent spots in membrane ruffles, as well as filopodia, where both proteins are known to be colocalized (Supplemental Fig. 1).

**FIGURE 5.** In vivo and in vitro association of fascin1 with LC3. (A) Coimmunoprecipitation of LC3 and fascin1. Lane 1, LAMP1 immunoprecipitate as a control; lane 2, LC3 immunoprecipitate. Immunoprecipitates were analyzed by Western blotting with anti-LAMP1, anti-fascin1, and anti-LC3 Abs. An asterisk in lane 2 is a cross-reactive IgG band. (B) In vitro GST pull-down assay to show direct binding between LC3 and fascin1. GST alone (lane 2) and GST-LC3 (lane 3) bound to glutathione Sepharose beads were incubated with nonfusion, recombinant fascin1, and bound fractions were analyzed by Western blotting with anti-fascin1 and anti-LC3 Abs. Lane 1, input; lane 4, GST-LC3 without fascin1 incubation showing no fascin1 is present in the original GST–LC3 preparation. (C) PLA. (a–c) Wild-type DCs; (d–f) fascin1 KO DCs. (a, d) Phase-contrast image; (b, e) fluorescent signals showing close association between fascin1 and LC3 by PLA (see Materials and Methods for detail); (c, f) merged images. Cell peripheries were marked by dashed lines. Note that fascin1 KO DCs show no PLA signals, confirming that fluorescent signals detected in wild-type DCs indeed indicate the association between fascin1 and LC3.

The LC3–fascin interaction may facilitate autophagy of cytoplasmic bacteria. We thus examined whether fascin1 alters the extent of LC3–L. monocytogenes association, an indication of autophagy-mediated bacterial trapping. Before infection, both wild-type and KO DCs show very few LC3 puncta (Fig. 6Aa, 6Ac). At 40 min postinfection, L. monocytogenes in wild-type DCs (indicated by arrowheads in panel b–d) were found to be much more frequently associated with LC3 than were L. monocytogenes in fascin1 KO DCs (indicated by arrowheads in Fig. 6Af–h). Quantitative measurements of LC3-positive L. monocytogenes at 20, 40, and 60 min revealed that wild-type DCs exhibited much faster kinetics of LC3–L. monocytogenes association than did fascin1 KO DCs (Fig. 6B). The fraction of LC3-positive bacteria in wild-type DCs increased from 23 to 47% from 20 to 40 min and leveled off at 60 min. In contrast, the fraction of LC3-positive L. monocytogenes in fascin1 KO DCs was just increased from 7% at 20 min to 16% at 40 min and 25% at 60 min. These results suggest a possibility that L. monocytogenes is processed via autophagy with a faster kinetics in wild-type DCs than in fascin1 KO DCs.

The faster kinetics of LC3 association could occur if L. monocytogenes in wild-type DCs entered more quickly into the cytoplasm than those in fascin1 KO DCs. This was possible because fascin1 reduced phagosomal pH from 6.0 to 5.5 and because the activity of LLO, a pore-forming toxin protein required for cytoplasmic entry, becomes maximum at a phagosomal pH of 5.5 (36, 40).

**Fascin1 appears to promote L. monocytogenes–LC3 association**

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**FIGURE 6.** Fascin1 facilitates L. monocytogenes–LC3 association. (A) Before infection; (b–d, f–h), 40 min postinfection with a wild-type strain of L. monocytogenes. Arrowheads in b–d indicate that L. monocytogenes is surrounded with LC3 in wild-type DCs, whereas arrowheads in f–h show L. monocytogenes that is not associated with LC3. (B) Time-course analysis of L. monocytogenes–LC3 association. Wild-type DCs show L. monocytogenes–LC3 association to a greater extent and with faster kinetics. Error bars, SD. Representative result from three independent experiments.
It is also possible that the LC3–L. monocytogenes association described above may not be derived from cytoplasmic L. monocytogenes but from vacuolar L. monocytogenes in a compartment known as spacious Listeria-associated phagosomes (SLAPs): SLAPs have been shown to be associated with LC3 (38), although the SLAP assembly appears to occur at a later time point of 4 h in macrophages. To test these possibilities, we measured, by electron microscopy, what fractions of L. monocytogenes were cytoplasmic and whether SLAP-like vacuoles were observed. Wild and fascin1 KO DCs were infected with wild-type L. monocytogenes and fixed at 50 min postinfection, at which time wild-type and fascin1 KO DCs show difference in the LC3 and L. monocytogenes association.

Fig. 7 shows three typical images of wild-type L. monocytogenes: they represent L. monocytogenes in vacuoles (Fig. 7A), L. monocytogenes apparently escaping from vacuoles (Fig. 7B), and L. monocytogenes in the cytoplasm (Fig. 7C). Quantitative analyses to determine the fractions of these three types of L. monocytogenes in DCs (Fig. 7D) revealed that L. monocytogenes rapidly escaped from phagosomes in DCs and that there is no significant difference in cytoplasmic penetration between wild-type and fascin1 KO DCs: at 50 min postinfection, the fractions of cytoplasmic L. monocytogenes were ∼46% ± 9% for wild-type DCs and 40% ± 9% for fascin1 KO DCs. The fractions of L. monocytogenes escaping from phagosomes were 25 ± 4% for wild-type and 31 ± 6% for fascin1 KO DCs. Electron microscopy also revealed few SLAP-like vacuoles, which is defined by the presence of more than three L. monocytogenes in spacious vacuoles. This is consistent with the previous report that only 10% of macrophages exhibited SLAPs and the majority of L. monocytogenes is cytoplasmic in macrophages at 1 h postinfection (38).

Cytoplasmic penetration of L. monocytogenes was also examined by immunofluorescence analyses. DCs infected with L. monocytogenes for 1 h were labeled with an Ab against Arp2/3, a marker of cytoplasmic L. monocytogenes (Supplemental Fig. 2). We found that the Arp2/3 signals of L. monocytogenes were similar between wild-type and fascin1 KO DCs (43 ± 14%) and fascin1 KO DCs (44 ± 24%). In contrast, mutant strain L. monocytogenes (DP L-2319) that cannot escape from phagosomes were not labeled with the Arp2/3 Ab, confirming that L. monocytogenes labeled with the Arp2/3 Ab represent cytoplasmic L. monocytogenes. These observations are consistent with the electron microscopic analyses and suggest that the difference in phagosomal pH between wild and fascin1 KO DCs did not largely affect the cytoplasmic entry. Taken together, the faster kinetics of LC3-Lm association suggests that wild-type DCs show higher autophagy activity than fascin1 KO DCs.

Fascin1 is involved in promoting autophagy flux

To monitor autophagy dynamics, we examined whether wild-type DCs have a higher autophagy flux (a fusion process from autophagosomes to autophagolysosomes) than do fascin1 KO DCs. By expressing tFLC3 (LC3 tandemly tagged with GFP and RFP) (39), we measured whether fascin1 alters autophagy flux in LPS-matured DCs. As Fig. 8A and 8B show, wild-type DCs displayed more red LC3 puncta than did fascin1 KO DCs. Quantitative analyses (Fig. 8C) revealed that 70% of wild-type DCs showed red LC3, whereas most fascin1 KO DCs (70%) exhibited green puncta. These results indicate that tFLC3 is in the acidic compartments in wild-type DCs, suggesting that fascin1 may promote autophagosome–lysosome fusion by increasing autophagic flux.

Discussion

Our results suggest that fascin1 plays a role in innate immunity by facilitating eradication of L. monocytogenes in DCs. This novel role of fascin1 would also be critical for adaptive immunity because DCs have to survive infection to present pathogen information to T cells. The ability of fascin1 to protect DCs from L. monocytogenes infection is significant: fascin1 reduces a L. monocytogenes replication rate by 2 times during the 1- to 4-h period postinfection (Fig. 1). This reduction is comparable to that reported for ATG5, a major constituent of autophagy, on Shigella replication in MEFs (40). Because macrophages express no fascin1, our results would explain the previous report that macrophages are more susceptible to L. monocytogenes infection than DCs (11, 12).

Role of fascin1 in phagosomal acidification

Before infection, both wild-type and fascin1 KO DCs show a similar vacuolar pH from neutral to alkaline, which is consistent with the lack of fascin1 expression in immature wild-type DCs (13). Whereas L. monocytogenes infection acidifies phagosomal pH of both wild-type and fascin1 KO DCs, fascin1 expression resulted in further acidification (Fig. 3). It has been demonstrated that maturation activates the vacuolar proton pump (V-ATPase) by increasing the association between the V0 and V1 sectors of V-ATPase (41). Because the V1 sector of V-ATPase binds to actin.
filaments (42–45), it is possible that fascin1, via bundling actin filaments, could promote the V$_{0}$-V$_{1}$ association of V-ATPase on phagosomes, promoting V-ATPase activity, thus lowering phagosomal pH more efficiently and accelerating phagolysosomal fusion of phagosome-entrapped bacteria. We are in the process of testing this possibility.

Our observation of acidic phagosomal pH is in agreement with a recent report by Westcott et al. (12) that *L. monocytogenes*-engulfed phagosomes of DCs are acidic. There are, however, conflicting reports regarding phagosomal pH in DCs (46, 47). One group has reported that phagosomal pH in DCs is kept neutral to alkaline due to reactive oxygen species production by the recruitment of NOX2 to phagosomes. The authors claimed that alkaline pH is critical to prevent excessive degradation of possible Ag peptides and thus preserve Ag information for presentation to T cells (46). In contrast, a recent study has shown that phagosomal pH is acidified with a variety of phagocytosed particles, including latex beads, and that limited proteolysis occurs by NOX2-mediated modification of cathepsins in a pH-independent manner (47).

Regardless of the origin of the conflict about pH of the latex bead–engulfed phagosomes, *L. monocytogenes*-engulfed phagosomes must be acidic. Unlike latex beads used in the studies by Savina et al. (46), *L. monocytogenes* escapes into the cytoplasm from phagosomes. This escape necessitates acidic phagosomal pH because phagosomal escape requires the activity of LLO, a pore-forming toxin protein that is active only in an acidic environment (48). Perhaps the responses of DCs to *L. monocytogenes* are quite different from those to opsonized latex beads. We recognized, for example, that *L. monocytogenes* infection rapidly induced fascin1 expression to a maximum within 1 h, whereas LPS activation took as long as 7 h to induce fascin1 expression to a similar level (data not shown). This observation indicates that *L. monocytogenes* infection rapidly induces DC maturation by pattern recognition receptor engagement, whereas opsonized latex beads may keep DCs in an immature state for a prolonged time. It is worthy of note that the timing of cytoplasmic penetration of *L. monocytogenes* coincides with the timing of fascin1 expression, as well as that of acidification (Figs. 3, 4).

**Role of fascin1 in autophagy**

Our results suggest that fascin1 may promote autophagy. How does fascin1 play such a role? Fascin1–LC3 binding is likely to be involved. Intracellular cytoplasmic *L. monocytogenes* has been shown to evade autophagy-mediated killing in many types of cells, including fibroblasts, macrophages, and Madin-Darby canine kidney cells (49–52). *L. monocytogenes* uses the virulence factor, ActA, for the evasion (51–54), and the binding of ActA to Arp2/3 or VASP is essential for evading autophagy (52). It has been suggested that the binding of ActA with Arp2/3 or VASP results in coating of the surfaces of *L. monocytogenes* with cytoplasmic host proteins. This coating would displace intracellular *L. monocytogenes* as a cytoplasmic structure, thereby evading the autophagy machinery. It is possible that fascin1 could negate this disguise. As an ActA/Arp2/3/ VASP complex polymerizes F-actin on the surface of *L. monocytogenes*, fascin1 would recruit LC3 via its binding to F-actin. Such LC3 recruitment could facilitate autophagic recognition, blocking the autophagy evasion. We are in the process of testing this hypothesis.

Fascin1 has been reported to promote *L. monocytogenes* motility in vitro (24), which could increase cell-to-cell transmission of *L. monocytogenes*. However, we observed that wild-type DCs with high fascin1 expression have no *L. monocytogenes* infection, suggesting that increased *L. monocytogenes* motility, if it ever happens in DCs, does not facilitate cell-to-cell transmission of *L. monocytogenes*. Indeed, we found that cell-to-cell transmission (judged from the size of *L. monocytogenes*-infected DC colonies) is lower in wild-type DCs than in fascin1 KO DCs (Supplemental Fig. 3).

**Conclusion**

We have demonstrated that fascin1 contributes to survival of DCs against *L. monocytogenes* infection. Judging from the proposed roles of fascin1 in lowering phagosomal pH and facilitating autophagy, it is possible that fascin1 protects DCs from infection by other bacterial pathogens. Fascin is abundantly expressed in both *Drosophila* hemocytes and sea urchin coelomocytes, both of which are phagocytic defense cells, involved in the innate immune system of these organisms (55–58). These results suggest an evolutionarily conserved role in fascin in innate immunity, and it would be interesting to see whether fascin1 also protects these nonmammalian phagocytic defense cells from bacterial infection.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


