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The Actin Regulatory Protein HS1 Is Required for Antigen Uptake and Presentation by Dendritic Cells

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The hematopoietic actin regulatory protein hematopoietic lineage cell-specific protein 1 (HS1) is required for cell spreading and signaling in lymphocytes, but the scope of HS1 function in Ag presentation has not been addressed. We show that dendritic cells (DCs) from HS1−/− mice differentiate normally and display normal LPS-induced upregulation of surface markers and cytokines. Consistent with their normal expression of MHC and costimulatory molecules, HS1−/− DCs present OVA peptide efficiently to CD4+ T cells. However, presentation of OVA protein is defective. Similarly, MHC class I-dependent presentation of VSV8 peptide and GRP94/VSV8 complexes is defective. Analysis of Ag uptake pathways shows that HS1 is required for receptor-mediated endocytosis, but not for phagocytosis or macropinocytosis. HS1 interacts with dynamin 2, a protein involved in scission of endocytic vesicles. However, HS1−/− DCs showed decreased numbers of endocytic invaginations, whereas dynamin-inhibited cells showed accumulation of these endocytic intermediates. Taken together, these studies show that HS1 promotes an early step in the endocytic pathway that is required for efficient Ag presentation of exogenous Ag by DCs. The Journal of Immunology, 2011, 187: 000–000.

Dendritic cells (DCs) are highly specialized for presentation of Ags to naive T cells. DCs survey peripheral tissues, ingesting large volumes of material by receptor-mediated endocytosis, phagocytosis, and macropinocytosis (1–5). In the presence of inflammatory signals, DCs undergo a maturation process that results in diminished endocytosis of Ag, enhanced acidification of Ag processing compartments, redistribution of MHC molecules to the cell surface, upregulation of costimulatory molecules, and increased cell motility. As they mature, DCs migrate to lymphoid organs, where they present peptides derived from non-self Ags to T cells, initiating an adaptive immune response.

Many of these aspects of DC function rely on actin and its regulatory proteins. During endocytosis, actin polymerization produces forces that promote internalization of plasma membrane vesicles. This is particularly obvious for phagocytosis and macropinocytosis, which involve large actin-rich cell surface protrusions (6–8). However, receptor-mediated endocytosis is also dependent on actin filaments, which work together with clathrin and other proteins such as dynamin 2 to drive the internalization of plasma membrane vesicles (9, 10). After vesicle internalization, the actin cytoskeleton serves as a highway to transport vesicles to compartments where Ag is processed, loaded onto MHC molecules, and transported back to the cell surface for recognition by T cells (1, 2, 11).

Macropinocytosis and phagocytosis depend on the Rho family GTPases CDC42 and Rac (12–14), and diminished uptake through these pathways in mature DCs has been linked to downregulation of CDC42 function (13). Notably, however, receptor-mediated endocytosis is not dependent on Rho GTPases, nor is it downregulated in mature DCs (13). In keeping with this finding, recent analysis has shown that mature DCs take up Ags efficiently via receptor-mediated endocytosis (15), a process that may be very important for presentation of Ags by lymphoid-resident DCs (16). Receptor-mediated Ag uptake by both immature and mature DCs is likely to be particularly important at low Ag dose. In addition to playing an important role in normal immune responses, the ability of DCs to take up material by receptor-mediated endocytosis has been widely exploited to target these cells for therapeutic purposes (e.g., Refs. 17–21).

Defects in actin regulatory proteins have far-reaching effects on DC function. Mutations in Wiskott–Aldrich syndrome protein (WASP) and its binding partner WASp interacting protein (WIP) lead to defects in Ag uptake, migration, and immunological synapse formation (22–27). We have recently found that WASp and WIP interact in DCs with a third protein, hematopoietic lineage cell-specific protein 1 (HS1; also called HCLS1, LckBP1) (28). HS1 is the hematopoietic lineage-specific homolog of the more...
widely expressed protein cortactin (29), and we have shown that HS1 is the only cortactin family member expressed in murine bone marrow-derived dendritic cells (28). Like WASp, HS1 can activate the Arp2/3 complex to drive the formation of branched actin filaments (30, 31). However, HS1 also binds to F-actin and stabilizes the branched actin network generated by WASp and other proteins (32). HS1 is a modular protein, with an N-terminal domain that binds to the Arp2/3 complex followed by a repeat region that binds to actin filaments (30, 31, 33, 34). The C-terminal half of the protein functions as a signaling adapter and consists of an extended proline-rich region and a C-terminal SH3 domain. The proline-rich region contains sites for immunoreceptor-induced tyrosine phosphorylation (35–37) and provides docking sites for several SH3 and SH2 domain-containing proteins, including Lck, Vav1, PLCγ1, and Itk (38–40). The HS1 SH3 domain mediates binding to the WIP/WASp heterodimer (28). The SH3 domain of cortactin also binds to dynamin 2 (41), though this interaction has not been shown for HS1.

The full scope of HS1 function within leukocyte lineages is not known. Mutations and polymorphisms in HS1 are associated with autoimmune disease (42, 43), though the mechanistic basis for this association has not been determined. Ablerrant HS1 expression and/or phosphorylation in B cells are associated with chronic lymphocytic leukemia (44–46). Deficiency for HS1 is associated with disruption of actin responses and Ca2+ signaling events leading to IL-2 promoter activation in T cells (39, 40), with defects in activation-induced cell death in B cells (47, 48), and with defects in chemotaxis and cytosis in NK cells (49).

In comparison with lymphocytes and NK cells, much less is known about HS1 function in professional APCs. As we recently showed, wild-type (WT) murine bone marrow-derived dendritic cells (BMDCs) do not express cortactin, and DCs from HS1−/− mice lack both cortactin family members (28). These cells exhibit defects in podosome organization and lamellipodial protrusion, resulting in a diminished directional persistence during chemotaxis. Because several studies have demonstrated a role for cortactin in endocytosis (50–56), we asked whether HS1 expression is required for Ag uptake and presentation by DCs. Our results demonstrate that HS1 expression is essential for efficient Ag processing and presentation to T cells and that this is due, at least in part, to a requirement for HS1 in receptor-mediated endocytosis of protein Ags.

Materials and Methods

Mice

HS1−/− mice (48) were back-bred across onto C57BL/6J or BALB/c backgrounds. DO-11.10 mice, which express a TCR specific for chicken OVA323–339/I-Ab, and C57BL/6J mice were obtained from The Jackson Laboratory. OTII mice, which express a TCR specific for chicken OVA323–339/I-Ab, were obtained from the National Institute of Child Health and Human Development. All studies involving animals were reviewed and approved by the Children’s Hospital of Philadelphia Institutional Animal Care and Use Committee.

Cell culture

Primary cultures of BMDCs were prepared as previously described (57). Briefly, bone marrow progenitor cells were flushed from femurs and tibia of 2- to 4-mo-old mice, and cells were cultured in IMDM supplemented with 10% FBS, recombinant mouse GM-CSF, glutamine, 2-mercaptoethanol, and penicillin/streptomycin. Medium was replaced every 2 d, and cells were used between 6 and 8 d of culture, when >70% of the cells are CD11c+.

Maturation of BMDCs was induced by culturing for 16–24 h in the presence of 100 ng/ml LPS (Escherichia coli 055:B5; Sigma) or, where indicated, 500 nM CpG (ODN 1668) or control oligonucleotides (InvivoGen).

Primary CD4+ T cells from DO-11.10 or OTII mice were prepared as described previously (40). Briefly, CD4+ T cells were enriched from spleens and lymph nodes by negative selection with anti-CD8 and anti-MHC class II using anti-rat IgG magnetic beads (Qiagen). The T cell hybridoma N15, which expresses a TCR specific for VSV/H2-Kb (58), was obtained from Dr. E. Reinherz (Dana-Farber Cancer Institute, Boston, MA) and cultured in RPMI 1640, 10% FCS, 0.1 mg/ml G418, and 0.2 mg/ml hygromycin. N15 cells were incubated overnight with PMA (10 ng/ml) prior to use in Ag presentation assays, a treatment necessary for optimal activation (58). Maximal activation of N15 cells was measured by cross-linking with plate-bound anti-CD3.

Analysis of BMDC maturation

For flow cytometry, BMDCs were incubated with rat anti-mouse CD16/CD32 (clone 2.4G2) to block FcγR, followed by staining with the following mAbs (all from BD Biosciences): allophycocyanin-conjugated hamster anti-mouse CD11c, FITC-conjugated hamster anti-mouse CD40, PE-conjugated rat anti-mouse mAbs to CD80, CD86, and MHC class I and class II. Nonspecific staining was defined using isotype-matched Abs. Cells were analyzed on a FACSCalibur (BD Biosciences) using FlowJo software (Tree Star). To measure TNF-α production, BMDC culture supernatants were harvested at the indicated times and assayed by ELISA (R&D Systems).

Protein binding studies and Western blotting

For GST-pulldown studies, recombinant GST-tagged SH3 domain of HS1 (aa 415–486) and a variant bearing an inactivating mutation (W456Y) were generated in bacteria as described (28). BMDCs were lysed in Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl pH 8, 1% NP-40, 100 mM NaCl, 5 mM EDTA, 0.5 mM CaCl2, protease inhibitors, 1 mM Na3VO4, and 5 mM NaF). Lysates were clarified by centrifugation and separated on a gel to obtain Glutathione resin-bound GST fusion proteins for 2 h. Beads were washed in NP-40 lysis buffer, eluted, and analyzed by Western blotting. For coimmunoprecipitation studies, 293T cells were transiently transfected with FLAG-tagged HS1 (FLAG.HS1 FL), an N-terminal deletion mutant lacking amino acids 1–335, which contain the Arp2/3 complex and actin binding regions (FLAG.HS1ΔN) or a variant of FLAG.HS1 ΔN bearing the W456Y mutation (FLAG.HS1ΔN W456Y) (41). In addition, cells were transfected with either enhanced GFP-tagged dynamin 2 (GFP.Dyn2 FL) or a dynamin 2 mutant lacking the proline-rich domain (GFP.Dyn2 ΔPRD) (41) (gifts from Drs. H. Cao and M. McNiven, Mayo Clinic, Rochester, MN). Cells were lysed in NP-40 lysis buffer, and HS1 was immunoprecipitated using M2 anti-FLAG agarose (Sigma).

For Western blotting, proteins were separated by SDS-PAGE, transferred to nitrocellulose, and blocked in 3% BSA in PBS. Blots were probed with primary Abs as indicated. Rabbit anti-mouse HS1 was described previously (28, 40). Goat anti-dynamin 2 (C-18) and rabbit anti-WIP (H-224) were from Santa Cruz, rabbit anti-WASP was from Upstate, rabbit anti-GFP was from Invitrogen, and mouse anti-GAPDH (6c5) was from EMD Chemicals. Primary Abs were detected with secondary Abs coupled to IRDye 800 (Rockland) or Alexa Fluor 680 (Invitrogen) and visualized using an Odyssey Imager (Lior). Quantitation was performed within the linear range.

Macropinocytosis and phagocytosis assays

To measure macropinocytosis, BMDCs were washed and resuspended at 8 × 10^6/ml in IMDM containing 0.5% BSA. Cells were mixed with prewarmed FITC-dextran (70,000 m.w.; Invitrogen) or Lucifer yellow (Invitrogen) at 1 mg/ml final concentration and incubated at 37°C for the indicated times. After washing three times with cold FACS buffer (5% FBS, 1 mM EDTA in PBS), internalized tracers were measured by flow cytometry. Time points were performed in duplicate, and mean fluorescence intensity values were calculated.

To analyze phagocytosis, BMDCs were plated on coverslips in 6-well plates and cultured overnight. Media was replaced with ice-cold media containing 25 μg/ml FITC–zymosan (Invitrogen) and incubated for 1 h at 4°C. After washing three times with cold media, prewarmed media was added to initiate uptake. Cells were incubated at 37°C for the indicated periods and then fixed in 3% paraformaldehyde/PBS. Cells were permeabilized with 0.3% Triton X-100, blocked with 0.05% saponin/1.25% fish skin gelatin in Tris buffered saline, and labeled with Alexa Fluor 594–phalloidin or with anti-LAMP1 (1D4B) followed by Alexa Fluor 647 anti-Rat Ig. The 1D4B mAb developed by Dr. J.T. August was obtained from The Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa Department of Biology (Iowa City, IA). Cells were imaged using a Leica SP2 confocal microscope. Zymosan particles were scored as internalized if they were surrounded by phalloidin or LAMP1 staining.
Ag presentation assays

VSV8 peptide (RGYVYQGL) from the vesicular stomatitis virus N protein and OVA323-339 peptide (ISQAVHAAHAEINEAGR) from chicken OVA were synthesized at the University of Chicago peptide facility, purified by HPLC, and verified by mass spectrometry. OVA protein was purchased from Sigma-Aldrich. OVA peptide was further purified by reverse-phase high performance liquid chromatography to ensure removal of containing peptides and LPS. For phagocytosis studies, latex beads (polystyrene 3-μm microspheres; Polysciences) were freshly coated by overnight incubation with the indicated ratios of OVA and BSA, maintaining a concentration of 10 mg/ml total protein. Complexes of the VSV8 peptide and the chaperone glucose regulated protein of 94 kDa (GRP94) were generated as described in Ref. 57. The N-terminal portion of GRP94 (N-355), is sufficient to mediate peptide binding, receptor-mediated uptake by DCs, and cross-presentation of peptide Ags to T cells (57). N-355 was expressed in SP9 cells and purified by affinity and size exclusion chromatography under LPS-free conditions as detailed in Vogen et al. (59). Peptide–GRP94 complex was prepared as previously described (57, 59). Briefly, N-355 and VSV8 were mixed in buffer A (20 mM HEPEs, pH 7.2, 150 mM NaCl, 10 mM KCI, 1 mM MgCl2 and 0.1 mM CaCl2), heat shocked for 10 min at 50 °C and then incubated at room temperature for 30 min. The complex was separated from unbound peptide by size exclusion chromatography.

To measure Ag presentation on MHC class II molecules, BMDCs were harvested and replated at 5 × 105 cells/well in flat-bottom 96-well plates in quadruplicate. Cells were then pulsed with either OVA protein or OVA323-339 peptide at the indicated concentrations for 4 h. To control for contaminating peptide in the OVA protein, some DCs were pre-fixed with paraformaldehyde prior to addition of protein. Where indicated, mannose receptors were blocked by incubation with 3 mg/ml mannan from Saccharomyces cerevisiae (Sigma) for 1 h prior to addition of soluble OVA. For presentation of phagocytosed OVA, cells were incubated for 4 h with OVA/BSA-coated beads at a ratio of 60 beads per cell. Ag-pulsed DCs were washed and cocultured with 2 × 105 CD4+ T cells for 24 h. Cells and supernatants were lysed together, and IL-2 was measured by ELISA (R&D Systems). Presentation on MHC class I molecules was performed similarly, except that BMDCs were pulsed with N-355–VSV8 complex or free VSV8 peptide for 3–4 h, and then an equal number of N15 hybrido cells was added to each well. IL-2 released after 24 h was measured by ELISA.

Biochemical analysis of receptor-mediated endocytosis

Seventy-five to one hundred micrograms of GRP94 N-355, mouse transferrin, or OVA (US Biological) were each labeled with 1.0 mCi[125I]iodine (Amersham) in Tris buffered saline, pH 7.2, using IODODEADS (Amerham) according to the manufacturer’s recommendations. The unbound[125I]iodine was removed on Biogel P10 columns (Bio-Rad, Hercules, CA). Final sp. act. was 2000–6000 cpm/ng protein. To assay receptor-mediated endocytosis, BMDCs were harvested, washed with HBSS, 0.1% BSA, and adjusted to 1 × 106/ml. Cells were then loaded with[125I]GRP94, [125I]transferrin, or [125I]OVA (10 μg/ml) for 1 h at 4 °C. After washing with ice-cold HBSS/BSA, cells were resuspended in the same buffer, and aliquots of 1 × 106 cells each were warmed to 37 °C for the indicated times before returning to 4 °C. Surface-bound transferrin was stripped by mixing cells with an equal volume of acid (0.25 M acetic acid, 0.5 M NaCl, pH 2.3) for 6–10 s, followed by immediate neutralization with 50 μl 1 M sodium acetate, pH 8. Surface-bound GRP94 or OVA was stripped by treatment with 1.25 mg/ml pronase (Roche) in DMEM for 45 min at 4 °C. After stripping, cells were washed, and cell-associated radioactivity was measured on a gamma-counter.

Electron microscopy

WT and HS1+/− BMDCs were grown on 35-mm tissue culture dishes. To inhibit dynamin, some dishes were treated with 80 μM of the dynamin inhibitor dynasore monohydrate (Sigma) for 60 min at 37 °C in serum-free media. Cells were then washed with HBSS and fixed as monolayers with 2.5% paraformaldehyde, 0.1 M sodium cacodylate, pH 7.4, containing 1.25 mM MgCl2 and 1.25 mM CaCl2. Cells were washed with 100 mM sodium cacodylate, postfixed in 2% aqueous OsO4 followed by 2% aqueous uranyl acetate, and dehydrated in graded EtOH. Dishes were then processed using a modification of Griffiths et al. (60) to release cell monolayers and a thin layer of polystyrene, and cell sheets were embedded in EPON-812 (Electron Microscopy Sciences). Ultrathin cross-sections of the cell sheets were contrasted with aqueous 1.5% uranyl acetate followed by Reynold’s lead citrate and viewed using a JEOL 1011 electron microscope operated at 100 kV. Images were acquired with an Orius CCD camera using Digital Micrograph software (Gatan).

To label receptor-mediated endocytic compartments, cells were allowed to internalize 50 μg/ml mouse transferrin–HRP (Thermo Scientific) for 15 min at 37 °C. To label the fluid-phase pathway, cells were allowed to internalize 15 mg/ml soluble HRP (Sigma) for 30 min at 37 °C. In both cases, cells were washed and fixed with glutaraldehyde as described previously. After fixation, cells were incubated for 30 min with 1 mg/ml 3,3′-diaminobenzidine (DAB; Sigma) and 0.01% H2O2 in 200 mM sodium cacodylate, pH 7.4. Cells were then washed with 100 mM sodium cacodylate and incubated with 2% aqueous OsO4 to generate an electron-dense HRP–DAB reaction product and postfix the cells. Subsequent processing was as described earlier.

To determine the number of endocytic invaginations, cell profiles were examined at random at ×60,000 or higher magnification, and structures were counted by an individual blinded to experimental conditions. Shallow, shallow pits were distinguished from non-pit regions of the membrane on the basis of clearly defined edges, relatively regular size, and, in some cases, an obvious clathrin coat. Pits and wide-neck invaginations were collectively defined as structures where the width of the structure at the plasma membrane was larger than its depth. Narrow-neck invaginations were defined as structures where the width of the mouth was less than the diameter of the invagination. Structures of an unclear nature were excluded from analysis. To determine density of these structures as a function of plasma membrane length, low-magnification (>5000) micrographs were captured, and the total length of the plasma membrane in each cell profile was measured using ImageJ v1.42 (National Institutes of Health). Filopodia were excluded from analysis, as these were difficult to measure accurately and were almost never observed to have endocytic invaginations.

Results

HS1 is not required for differentiation or maturation of DCs

One of the key changes occurring during DC maturation is the reprogramming of actin regulatory pathways (13, 61). This involves changes in expression patterns of actin regulatory proteins (62, 63) and functional downregulation of Rho family GTPases that drive the formation of branched actin filaments. We recently showed that DCs express HS1, a protein that promotes the formation of branched actin filaments in a Rho GTPase-independent manner (28). To ask whether HS1 expression is also downregulated upon DC maturation, we cultured BMDCs from WT mice for 24 h with LPS or CpG DNA to induce maturation and analyzed by Western blotting for HS1. As shown in Fig. 1A and 1B, mature DCs do not downregulate HS1 expression. Instead, these cells express ∼2-fold more HS1 than immature DCs, indicating that HS1 expression is upregulated as part of the maturation program.

We showed previously that BMDCs from HS1+/− mice lack both HS1 and the related protein cortactin (28). To ask whether HS1 is required for DC development and/or maturation, BMDCs generated from WT and HS1+/− mice were cultured for 24 h with or without LPS or CpG DNA, and surface marker expression was analyzed by flow cytometry. Although the profiles of individual surface markers varied somewhat between DC preparations, cultures from HS1+/− and WT mice generated equivalent numbers of CD11c+ cells, and maturation-induced upregulation of MHC molecules and costimulatory proteins was similar in the two populations (Fig. 1C and data not shown). Secretion of TNF-α was also comparable in the two populations of cells (Fig. 1D). Taken together, these results show that HS1 is not required for development or maturation of BMDCs.

HS1 is required for presentation of protein Ags

We next asked whether HS1 is required for Ag presentation. As shown in Fig. 2A, HS1+/− DCs could present OVA peptide efficiently to CD4+ T cells, as measured by IL-2 production by T cells from DO-11.10 mice. However, if HS1+/− DCs were pulsed instead with intact OVA protein, they activated T cells much less efficiently than WT DCs. Although Ag insolubility precluded our ability to reach plateau levels of T cell activation, comparison of the Ag dose-
HS1-dependent Ag presentation in dendritic cells

Response shows that HS1−/− DCs required 30- to 100-fold more protein than WT DCs to achieve comparable levels of T cell activation (Fig. 2B). Similar results were obtained with OTII cells and HS1−/− BMDCs on the C57BL/6 background (Fig. 2C). To analyze Ag presentation on MHC class I molecules, we took advantage of our experience working with the endoplasmic reticulum chaperone GRP94. We showed previously that GRP94 binds to various peptides, and the complex is taken up by DCs through a receptor-mediated process and transported to degradative organelles (57). GRP94-bound peptides then escape the endocytic pathway and are loaded onto MHC class I molecules in the endoplasmic reticulum. As shown in Fig. 2E and 2F, HS1−/− DCs presented VSV8 peptide efficiently to the H2-Kb–restricted hybridoma N15, whose TCR is specific for VSV8. However, presentation of GRP94–VSV8 peptide complexes to this hybridoma was 10-fold less efficient than presentation by WT DCs. Taken together, these results indicate that HS1 is required for the uptake or processing of protein Ags, and thereby for their presentation, but is not needed for later events leading to productive T cell activation.

HS1 is required for efficient receptor-mediated endocytosis

DCs take up Ag by multiple mechanisms, including phagocytosis, macropinocytosis, and receptor-mediated endocytosis. Because phagocytosis and macropinocytosis are heavily dependent on actin polymerization to mobilize large pseudopodial extensions, we first asked whether HS1 is required for Ag uptake through these pathways. To assess macropinocytosis, immature WT and HS1−/− DCs were allowed to take up FITC–dextran (Fig. 3A) or Lucifer yellow (Fig. 3B), for various times, and uptake was assessed by flow cytometry. In both cases, the time course of fluid phase uptake was identical in control and HS1-deficient cells. To ask whether the maturation-associated downregulation of macropinocytic activity is intact in HS1−/− DCs, mature DCs were generated by culturing overnight in the presence of LPS. As reported previously (13), WT DCs showed diminished fluid-phase endocytosis upon maturation (Fig. 3B). Mature HS1−/− DCs (open squares) behaved similarly, indicating that HS1 is not required for reprogramming of the macropinocytic pathway.

To test the role of HS1 in phagocytosis, WT and HS1−/− DCs were incubated with FITC-conjugated zymosan particles at 4°C, warmed to 37°C to allow uptake, and analyzed by immunofluorescence microscopy. As shown in Fig. 3C, both WT and HS1−/− DCs exhibited F-actin–rich phagocytic cups at early times of internalization. To assess the kinetics of phagocytosis, cells fixed at various times after warming were labeled with fluorescent phalloidin to define cell boundaries, and the percentage of cells with...
FIGURE 2. HS1 is required for presentation of protein Ags. A and B, BMDCs cultured from WT and HS1/−/− mice on the BALB/c background were pulsed with the class II-restricted OVA253–264 peptide (A) or with the whole OVA protein (B) at the indicated doses for 4 h. They were then cocultured with OVA-specific DO-11.10 T cells, and 24 h later, IL-2 levels in the culture supernatants were measured by ELISA. C, BMDCs cultured from WT and HS1/−/− mice on the C57BL/6 background were incubated in the presence or absence of mannan to block mannose receptors, and then OVA protein was added, so that any residual uptake would be by macropinocytosis. Ag presentation to OTII T cells was then assayed as in A and B. In this experiment (representing two of four replicates), mannan completely blocked the response, indicating that even very high doses of OVA are taken up almost exclusively by receptor-mediated endocytosis. C, WT DCs without mannan; □, HS1/−/− DCs without mannan; Δ, WT DCs with mannan; ●, HS1/−/− DCs with mannan. Inset, Data from a separate experiment (representing two of four replicates) where T cell responses to mannan-blocked DCs were measurable at high OVA doses. Open bars, WT DCs with mannan; filled bars, HS1/−/− DCs with mannan; differences between WT and HS1/−/− DCs in the presence of mannan were not statistically significant at any dose of OVA. D, WT and HS1/−/− BMDCs (C57BL/6 background) were allowed to phagocytose latex beads coated with OVA and serum albumin in the indicated ratios, holding total protein and bead number constant. OTII T cell responses were then measured. E and F, WT and HS1/−/− BMDCs were pulsed with free VSV8 peptide (E) or VSV8 peptide prebound to GRP94 (F) at the indicated doses for 4 h. VSV8-specific N15 T hybridoma cells were incubated with Ag-pulsed BMDCs for 24 h, and IL-2 levels in the supernatants were measured by ELISA. Data represent means ± SD from replicate wells of one representative experiment.

internalized FITC–zymosan was determined. As shown in Fig. 3D, the kinetics of zymosan internalization were unaffected by HS1 deficiency. Moreover, labeling for the lysosomal protein LAMP1 showed that internalized particles were transported to lysosomal compartments with normal kinetics (data not shown). Similarly, phagocytosis of apoptotic cell debris or of fluorescent *Listeria* were not impaired in HS1/−/− DCs (data not shown). Taken together, these studies show that HS1 is not required for macrophagocytosis or phagocytosis by DCs.

We next asked whether receptor-mediated endocytosis is dependent on HS1. Initially, we tested endocytosis of transferrin, with is taken up by transferrin receptors via clathrin-coated vesicles (64). To obtain a quantitative measure of transferrin internalization, WT and HS1/−/− DCs were surface-labeled with [125I]transferrin at 4°C, washed, and warmed to 37°C for various times. Residual surface-bound transferrin was then removed by washing at low pH, and cell-associated radioactivity was determined. As shown in Fig. 4A, HS1−/− DCs internalized transferrin at only 30–40% of the efficiency of WT cells. The diminished uptake was apparent both in terms of the rate of uptake and the overall amount of transferrin taken up. Although diminished ligand uptake can theoretically reflect either reduced endocytosis or enhanced ligand recycling, starting transferrin receptor levels were similar in WT and HS1−/− DCs, and the initial rates of endocytosis were already significantly different, arguing against a significant contribution from differential recycling. Thus, we conclude that the diminished overall uptake of transferrin by HS1−/− DCs reflects a defect in initial endocytic internalization. OVA is taken up in a receptor-mediated fashion by the mannose receptor (65, 66), and GRP94 is taken up by CD91 and other scavenger receptors (57, 67–70). To ask whether HS1 deficiency also leads to reduced endocytosis of these proteins, we analyzed their internalization using the same assay. As shown in Fig. 4B and 4C, uptake of both OVA and GRP94 was significantly reduced in HS1−/− DCs, indicating that the requirement for HS1 is a general one.

As an independent means of assessing endocytosis, HS1−/− DCs were incubated for 15 min in the presence of transferrin-HRP. Cells were then fixed and processed for cytochemistry and electron microscopy. As shown in Fig. 5A, WT BMDCs showed numerous vesicles and tubular endosomes filled with HRP reaction...
By comparison, HS1−/− BMDCs contained far fewer HRP+ endocytic structures (Fig. 5B). Indeed, most cell profiles lacked labeled endosomes; the image shown in Fig. 5B was selected because it contains one rare HRP-positive organelle (arrowhead). Importantly, HS1−/− DCs took up free HRP by fluid-phase endocytosis as efficiently as WT cells. As shown in Fig. 5C and 5D, both cell types exhibited numerous macropinosomes containing electron-dense reaction product. Together with the biochemical analysis of ligand internalization, these data show that HS1 is specifically required for efficient uptake of ligands via receptor-mediated endocytosis into early endocytic compartments.

On the basis of our finding that HS1 is selectively required for receptor-mediated endocytosis in DCs, we asked whether the Ag presentation defects in HS1−/− DCs are limited to Ags taken up via this pathway. To facilitate comparisons, we targeted the same protein, OVA, for uptake via different endocytic mechanisms. First, cells were incubated with soluble OVA in the presence of excess mannan to inhibit receptor-mediated endocytosis and drive fluid-phase uptake. As shown in Fig. 2C, mannan treatment strongly inhibited Ag presentation, confirming that OVA is taken up by receptor-mediated endocytosis, even at very high doses. In two of four replicate experiments, no residual Ag presentation could be detected, making it impossible to assess the effects of HS1 deficiency on presentation of OVA taken up by fluid-phase endocytosis. In two of four experiments, although T cell activation in response to mannan-treated DCs was low (∼10% of that observed in the absence of mannan), it was measurable at the highest doses of OVA (Fig. 2C, inset). In such experiments, WT and HS1−/− DCs activated T cells equally well at all doses of OVA where a response could be measured. We conclude that HS1−/− DCs are competent to present OVA taken up by fluid-phase endocytosis. We also tested the presentation of phagocytosed OVA using latex beads coated with varying ratios of OVA protein and serum albumin. As shown in Fig. 2D, the response to phagocytosed OVA was similar in T cells activated by WT and HS1−/− DCs. Taken together, these studies show that the requirement for HS1 in presentation of protein Ags is linked to its role in receptor-mediated endocytosis and that HS1 is not needed for presentation of Ags taken up by other routes.

**HS1 binds to dynamin 2 and promotes formation of plasma membrane invaginations**

The HS1 homolog cortactin has been shown to interact through its SH3 domain with several proteins that promote endocytosis, including WASp, WIP, and dynamin 2 (41, 52, 71). In particular, cortactin interacts physically and functionally with dynamin 2 to drive the internalization of endocytic vesicles (51, 56, 72–75). We have shown that HS1 binds via its SH3 domain to the actin regulatory proteins WASp and WIP (28). To ask whether HS1 interacts in a similar way with dynamin 2, we probed DC lysates with the GST-tagged HS1 SH3 domain and blotted the bound proteins for dynamin 2. WASp and WIP were used as positive controls. As shown in Fig. 6A, all three proteins are associated with the HS1 SH3 domain. Mutation of tryptophan 465, a residue required for canonical binding to proline-rich ligands, disrupted binding, demonstrating the specificity of these interactions. To confirm that binding of HS1 and dynamin 2 takes place in intact cells and to map the relevant region of dynamin 2, we performed coimmunoprecipitation studies on cells coexpressing epitope-
tagged HS1 and dynamin 2 constructs. As shown in Fig. 6B, full-length HS1 and dynamin 2 could be coimmunoprecipitated from cells. This interaction does not require the Arp2/3- or actin-binding domains of HS1, as an N-terminal deletion mutant lacking these regions bound dynamin 2 efficiently. Indeed, on a mole-per-mole basis, this mutant showed ~200-fold more binding to dynamin 2 compared with that of full-length HS1. The enhanced binding ability of this HS1 truncation mutant also allowed detection of binding to endogenous dynamin 2 (Fig. 6B, “end”). Mutation of W465 within the SH3 domain of the HS1 DN mutant dramatically diminished dynamin 2 binding, confirming that the SH3 domain of HS1 mediates interaction with dynamin 2. Conversely, a dynamin 2 mutant lacking the C-terminal proline-rich domain (41) failed to coimmunoprecipitate efficiently with either full-length HS1 or the HS1 deletion mutant (Fig. 6B, “ΔPRD”). Low levels of association of HS1 and dynamin 2 were detectable under conditions where SH3–PRD interactions were abrogated, suggesting that a secondary interaction outside these domains may exist. Nonetheless, our results indicate that HS1 and dynamin 2 interact primarily through binding of the HS1 SH3 domain to the proline-rich domain of dynamin 2.

Dynamin is known to drive the scission of plasma membrane invaginations during receptor-mediated endocytosis, and cells expressing a dominant-negative dynamin mutant or treated with the dynamin inhibitor dynasore generate increased numbers of deep, wide-neck (U-shaped) and narrow-neck (O-shaped) endocytic invaginations (76). To ask whether HS1 is required at the same step in endocytic traffic, we analyzed plasma membrane invaginations in WT and HS1−/− BMDCs. Fig. 7A shows examples of shallow pits and wide-neck invaginations (top row), as well as narrow-neck invaginations (bottom row) found in WT cells. The number of these structures per linear micrometer of plasma membrane was determined as described in Materials and Methods. As anticipated, treatment of WT cells with dynasore resulted in a significant increase in the surface density of endocytic invaginations (Fig. 7B). These consisted primarily of deep invaginations with a wide neck. In contrast, HS1−/− DCs showed only about half as many endocytic invaginations as WT cells, and this
decrease was observed for both wide-neck and narrow-neck invaginations. These data indicate that HS1 and dynamin affect distinct steps of endocytosis in DCs and support a model in which an HS1-dependent step occurs early in the formation of membrane invaginations, whereas dynamin is needed to complete later steps of membrane deformation and vesicle scission.

**Discussion**

DC sentinel function depends on the ability of these cells to take up a wide variety of antigenic materials. This is achieved through a combination of phagocytosis, macropinocytosis, and receptor-mediated endocytosis (1, 3, 4, 11). We have found that HS1 plays an important role in this process by promoting receptor-mediated endocytosis of protein Ags. DCs from HS1−/− mice show diminished receptor-mediated endocytosis of multiple proteins and impaired presentation of exogenous protein-derived Ags on both MHC class I and MHC class II.

The requirement for HS1 in Ag trafficking and presentation appears to be restricted to receptor-mediated endocytic pathway. HS1−/− DCs presented peptide Ags efficiently, indicating that HS1 is not required for actin-dependent events at the DC side of the immunological synapse (27, 77–79). Moreover, although there is strong evidence that actin polymerization is important for macropinocytosis and phagocytosis (12, 14, 80), HS1 expression was not required for these processes. By extension, as we find no cortactin expression in murine BMDCs (28), cortactin family members generally are dispensable. Both macropinocytosis and phago-

**FIGURE 6.** The SH3 domain of HS1 interacts with the proline-rich domain of dynamin 2. A, GST alone, the SH3 domain of HS1 fused to GST, or the inactive SH3N→Y mutant fused to GST was immobilized on glutathione resin and incubated with DC lysates. Bound proteins were analyzed by SDS-PAGE and immunoblotting with anti-dynamin 2, anti-WASp, or anti-WIP. Total, protein expression in whole-cell lysates. B, 293T cells were transiently transfected with full-length GFP-tagged dynamin 2 (GFP.Dyn2 FL, black arrowheads) or a mutant lacking the proline-rich domain (ΔPRD, gray arrowheads), together with FLAG-tagged HS1 (FLAG.HS1 FL), an N-terminal deletion mutant of HS1 lacking the actin regulatory region (ΔN), or the ΔN mutant bearing a point mutation in the SH3 domain (ΔN^N→Y). Cells were lysed, immunoprecipitated with anti-FLAG, and Western blots were probed as indicated. Note that the ΔPRD mutant is not detected by the anti-dynamin 2 (which was generated against this C-terminal region of dynamin 2) but is readily detectable in the WCL using anti-GFP Ab. end, endogenous dynamin 2; *HS1 variants. WCL, whole-cell lysate from which immunoprecipitation was performed.

**FIGURE 7.** HS1 promotes endocytic invagination. A, Electron micrographs illustrating various stages in the process of coated vesicle formation. All images are from WT BMDCs; structures in HS1−/− BMDCs were comparable in morphology. Top row, Examples of shallow pits and wide-necked invaginations. Bottom row, Examples of narrow-neck invaginations. B, Quantitation of the frequency of the structures shown in A in WT cells, WT cells treated with 80 μM dynasore, and HS1−/− DCs. Cell profiles were photographed at random, and the number of structures in each category per linear micrometer of plasma membrane was determined as described in Materials and Methods. Values represent mean ± SEM from three to four independent experiments, totaling 66–68 cells and 100–500 pits or invaginations per experimental condition. ***p < 0.001 (relative to untreated WT cells).
cytosis are downregulated upon DC maturation (13, 65), whereas receptor-mediated endocytosis proceeds unabated in mature DCs (13, 15, 81). Phagocytosis and macrophagocytosis depend on the actin nucleating proteins WASp and WAVE, and the downregulation of these processes is linked to diminished activation of the Rho family GTPase CDC42 upstream of these events (13). Notably, HS1 differs from WASp and WAVE in that it does not depend on activation by Rho GTPases, and so is not subject to downregulation at this level. Indeed, we find that HS1 expression is modestly upregulated upon DC maturation. Moreover, HS1 is enriched in podosomes, which are largely dissolved on DC maturation (28), presumably increasing the pool of HS1 available to support endocytotic functions in mature DCs.

Our analysis of HS1−/− DCs shows a 2-fold decrease in the maximal uptake of endocytosed proteins, yet these cells require 30- to 100-fold more protein Ag to achieve the same level of T cell activation as that of WT DCs. The assay we used for receptor-mediated endocytosis measures only a single, synchronized round of protein uptake. When multiplied by the many rounds of endocytosis that take place within the 4-h period of the Ag pulse, the defects we observe could readily account for at least 30- to 50-fold reduction in overall Ag uptake. Thus, the requirement for HS1 to facilitate endocytosis is likely to be the primary cause of the diminished Ag presentation that we observe in HS1-deficient DCs. The finding that Ags taken up by phagocytosis and macrophagocytosis are presented efficiently further supports this conclusion. Nonetheless, it is possible that HS1 is also required for additional intracellular steps of Ag trafficking. Testing this possibility will require quantitative analysis of the fate of the endocytosed pool of protein.

Although the DC literature tends to focus on phagocytosis and macrophagocytosis, receptor-mediated endocytosis is also an important means of Ag uptake. Typically, it is argued that receptor-mediated endocytosis is particularly important at low ligand concentrations. While this is no doubt true, our side-by-side comparison of presentation of OVA-derived peptides after uptake by receptor-mediated endocytosis and macrophagocytosis shows that receptor-mediated uptake is dominant even at very high Ag doses. DCs express numerous cell surface receptors that preferentially bind non-self molecules and take them up in a highly efficient manner. OVA is taken up by mannose receptors (65, 66) (Fig. 2C), which is important for uptake of pathogen-derived glycoproteins (82–84). Uptake and cross-presentation of GRP94–peptide complexes also require receptor-mediated endocytosis, though the relevant receptor(s) remain controversial (57, 67–70).

Other receptors also play an important physiological role. Among these are the C-type lectin DC-SIGN, which promotes the endocytosis of HIV and other viruses and their presentation on MHC class I (85–88). The multilectin receptor DEC205 plays a similar role. Although the DC literature tends to focus on phagocytosis and macrophagocytosis, receptor-mediated endocytosis is also an important means of Ag uptake. Typically, it is argued that receptor-mediated endocytosis is particularly important at low ligand concentrations. While this is no doubt true, our side-by-side comparison of presentation of OVA-derived peptides after uptake by receptor-mediated endocytosis and macrophagocytosis shows that receptor-mediated uptake is dominant even at very high Ag doses. DCs express numerous cell surface receptors that preferentially bind non-self molecules and take them up in a highly efficient manner. OVA is taken up by mannose receptors (65, 66) (Fig. 2C), which is important for uptake of pathogen-derived glycoproteins (82–84). Uptake and cross-presentation of GRP94–peptide complexes also require receptor-mediated endocytosis, though the relevant receptor(s) remain controversial (57, 67–70).

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HS1 functions both as an actin regulatory protein and an adapter molecule, and it seems likely that both of these functions are called into play to promote receptor-mediated endocytosis. We show that HS1 interacts with dynamin 2 through binding of its C-terminal SH3 domain to the proline-rich domain of dynamin 2, the same mechanism used to mediate cortactin–dynamin 2 interaction (41). Notably, however, the endocytic phenotype of HS1−/− DCs does not mirror that of cells treated with the dynamin GTPase inhibitor dynasore. In keeping with studies in other cell types (76), we found that treatment of DCs with dynasore led to the accumulation of deep endocytic invaginations. The accumulation of wide-necked (U-shaped) structures was predominant, though narrow-necked (O-shaped) endocytic invaginations were also observed. The latter structures are also seen in cells expressing dominant-negative dynamin mutants and are thought to represent a requirement for dynamin in the fission of fully formed coated pits (9, 96). The U-shaped invaginations represent an earlier endocytic intermediate and are proposed to represent an additional requirement for dynamin in driving membrane deformation (76). In contrast to dynamin-inhibited cells, HS1-deficient DCs exhibited abnormally low numbers of both U-shaped and O-shaped endocytic invaginations. The simplest interpretation of this finding is that HS1 is required for an early step in membrane invagination, preceding the deep invaginations formed by dynamin activity. These early intermediates are thought to be metastable (97, 98), perhaps explaining why no clear accumulation of shallow pits was observed in HS1-deficient DCs. This interpretation is consistent with a two-step model for dynamin function, in which dynamin first serves as regulatory GTPase to ensure vectorial coat assembly and enhance membrane curvature, and subsequently, in an assembled state, uses GTPase-driven mechano-chemical activity to drive vesicle scission (96). According to this model, SH3 domain-containing proteins such as HS1 sense the status of cargo loading, membrane curvature, and/or coat assembly and regulate this functional switch.

In addition to binding dynamin 2, HS1 activates the Arp2/3 complex, binds actin filaments, and interacts with the actin regulatory proteins WASp and WIP (28, 30). Thus, it is also likely that HS1 promotes actin-dependent aspects of endocytosis. The literature regarding the requirement for actin polymerization in endocytosis is conflicting. In S. cerevisiae, actin recruitment occurs after clathrin coat formation and provides an essential force for membrane deformation (99). In mammalian cells, however, results are variable and depend on the cell type and the nature of the endocytic structure (100–103). Recent work from the Kirchhausen group (104–106) has shown that actin regulatory proteins are recruited to long-lived endocytic structures and are particularly important for internalization of large structures, such as clathrin plaques and some virus particles. On the basis of this work, it seems that actin polymerization provides the force needed to generate oversized clathrin-coated structures and to overcome other obstacles, such as high membrane tension (107). Because HS1 promotes the formation of branched-actin filaments, it may play a key role in this process. Importantly, the interactions of HS1 with the actin cytoskeleton and dynamin are likely to be coupled. Indeed, we find that interactions between HS1 and dynamin are dramatically enhanced by deletion of the N-terminal actin regulatory region of HS1. This and similar observations regarding HS1 binding to WASp and WIP (28) are compatible with a model in which the N terminus of HS1 partially restricts availability of the SH3 domain, such that interaction with the actin cytoskeleton releases the SH3 domain and promotes binding to dynamin 2 and other molecules. This model explains our inability to coimmunoprecipitate endogenous dynamin 2 and HS1, as the pool of...
active HS1 would be selectively associated with the insoluble actin cytoskeleton. A positive feedback loop may also be at play, as engagement of the dynamin proline-rich domain by SH3 domains has been shown to promote dynamin GTPase activity (108, 109). Such a mechanism would promote the coupled assembly of actin and dynamin at endocytic invaginations.

An important unresolved question is to what extent HS1 function is distinct from that of its more widely expressed homolog cortactin. In non-hematopoietic cells, the weight of the evidence indicates that cortactin plays a key role in endocytic uptake (51, 56, 72–75, 111), although some studies challenge this view (112, 113). Cortactin is recruited to long-lived clathrin-rich regions of the membrane, and kinetic analysis shows that cortactin recruitment peaks at a late time point, close to the time of dynamin recruitment (72, 104).

Furthermore, phosphorylation of cortactin promotes its association with dynamin (56, 74), and the tyrosine phosphorylation sites that have been linked to regulated cortactin function are conserved in HS1. Given all the biochemical and cell biological similarities between cortactin and HS1, why did hematopoietic lineages evolve a distinct family member? There are, in fact, some important functional differences between HS1 and cortactin. The structural features necessary for F-actin binding are distinct (31). Moreover, HS1 is less efficient than cortactin at driving Arp2/3 complex-nucleated actin polymerization (30). Actin binding promotes cortactin interaction with dynamin (74), and our evidence suggests that the same is true for HS1. Thus, the difference in mode of actin binding between HS1 and cortactin may translate into important differences in the mechanisms by which these proteins link the actin cytoskeleton to dynamin function. Because BMDCs from HS1−/− mice lack both HS1 and cortactin, they provide an ideal experimental system in which to carry out comparative reconstitution studies to address this possibility.

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Disclosures

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References


