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Developmental Control of Endocytosis in Dendritic Cells by Cdc42

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Summary

Dendritic cells (DCs) developmentally regulate antigen uptake by controlling their endocytic capacity. Immature DCs actively internalize antigen. However, mature DCs are poorly endocytic, functioning instead to present antigens to T cells. We have found that endocytic downregulation reflects a decrease in endocytic activity controlled by Rho family GTPases, especially Cdc42. Blocking Cdc42 function by Toxin B treatment or injection of dominant-negative inhibitors of Cdc42 abrogates endocytosis in immature DCs. In mature DCs, injection of constitutively active Cdc42 or microbial delivery of a Cdc42 nucleotide exchange factor reactivates endocytosis. DCs regulate endogenous levels of Cdc42-GTP with activated Cdc42 detectable only in immature cells. We conclude that DCs developmentally regulate endocytosis at least in part by controlling levels of activated Cdc42.

Introduction

Dendritic cells (DCs) are the most professional of all antigen presenting cells (APCs), uniquely able to stimulate even immunologically naïve T lymphocytes (Cella et al., 1997a; Banchereau and Steinman, 1998; Mellman et al., 1998). Their efficiency stems not only from the high levels of major histocompatability complex (MHC) molecules they express, but also from their capacity to produce peptide–MHC complexes from minute quantities of almost any antigen. In addition, DCs display high levels of plasma membrane proteins such as CD86 and CD40, which act together with MHC–peptide complexes in signaling T cell activation.

DCs exhibit a host of other cell biological specializations which further contribute to their efficiency as APCs. Among the most striking is their capacity to internalize large quantities of soluble or particulate antigen in a highly regulated fashion (Sallusto et al., 1995). DCs regulate virtually all aspects of antigen processing and presentation in a fashion that accounts for the distinct functions they serve at different stages of their life cycle. Derived from bone marrow precursors, DCs reside in peripheral tissues where they act as sentinels awaiting the arrival of infectious agents or other foreign antigens. These immature DCs are highly endocytic and thus efficient at antigen capture. However, they are poor APCs, because they retain most of their MHC molecules intracellularly and are unable to form peptide–MHC class II (MHC II) complexes (Cella et al., 1997b; Pierre et al., 1997). Inflammatory stimuli, such as LPS and TNF-α, trigger DC maturation resulting in the generation of peptide–MHC complexes, and the upregulation of surface MHC I, MHC II, CD40, and CD86. Maturation thus converts immature DCs from cells adapted for antigen accumulation to cells specialized for the processing and presentation of previously encountered antigens to T cells (Cella et al., 1997a; Mellman et al., 1998).

DC maturation is accompanied by carefully orchestrated alterations in membrane traffic. A number of novel mechanisms have been identified for controlling the expression of individual proteins at the plasma membrane, particularly in the case of MHC II molecules (Cella et al., 1997b; Pierre et al., 1997; Pierre and Mellman, 1998; Driessen et al., 1999). In immature DCs, MHC II-invariant (Ii) chain complexes are targeted from the Golgi to endosomes and then to lysosomes due to inefficient Ii chain cleavage in endosomes by the cysteine protease cathepsin S (Pierre and Mellman, 1998; Driessen et al., 1999). In mature DCs, cathepsin S activity is activated following the downregulation of an endogenous specific inhibitor of cathepsin S (cystatin C), resulting in more efficient Ii chain cleavage. This releases MHC II from Ii chain and thus its lysosomal targeting signal, allowing for the subsequent delivery of αβ dimers to the plasma membrane. Maturing DCs also possess a novel mechanism to recover previously synthesized MHC II molecules from lysosomes. Here, maturation appears to activate a selective sorting event involving the accumulation of MHC II-peptide into distinctive nonlysosomal transport vesicles (Turley et al., 2000).

Maturation also induces a dramatic inhibition of endocytosis. While immature DCs actively carry out macropinocytosis and phagocytosis, in mature DCs these activities are barely detectable (Inaba et al., 1993; Sallusto et al., 1995; Austyn, 1996). The loss of endocytic capacity greatly attenuates the ability of mature DCs to internalize antigen, thus limiting the range of antigens DCs will be able to present. Virtually nothing is known about the mechanisms that control endocytosis during DC maturation. It is increasingly clear, however, that actin and its regulation by Rho family GTPases play important roles in mediating phagocytosis and macropinocytosis. Engagement of phagocytic receptors induces the activation of Rho GTPases (Cox et al., 1997; Caron and Hall, 1998; Massol et al., 1998). Interestingly, a number of important pathogens, such as *Salmonella typhimurium* and *Shigella flexneri*, drive their own internalization by activating Rho GTPases, using a novel type III secretion apparatus to "inject" specific guanine nucleotide exchange factors into the cytoplasm of their intended host cells (Chen et al., 1996; Watarai et al., 1997; Hardt et al., 1998; Nhieu and Sansonetti, 1999). We have found that mouse DCs may make use of a similar strategy, controlling levels of activated Cdc42 to developmentally regulate their capacity for endocytosis.

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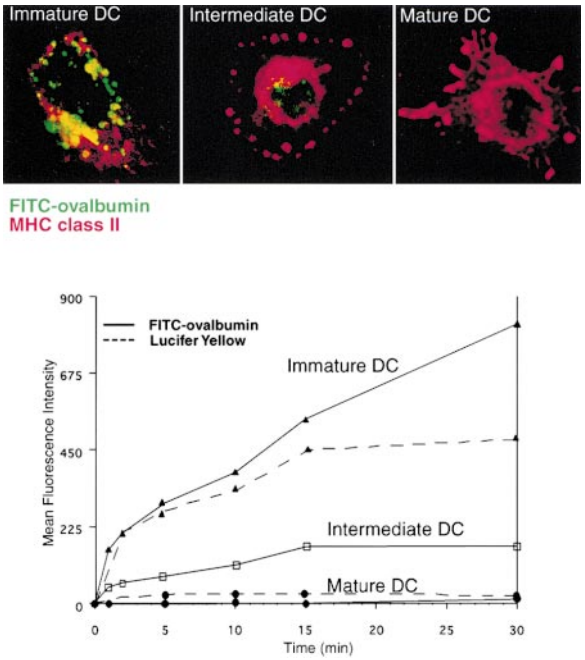


Figure 1. Endocytosis Is Rapidly Downregulated during Dendritic Cell Development

(A) Cluster-purified, synchronized cultures (Pierre et al., 1997) of immature, intermediate, and mature DCs on glass coverslips were pulsed with FITC-OVA for 30 min, and labeled with anti-MHC II β chain rabbit polyclonal antibody and a Texas red conjugated secondary goat anti-rabbit antibody. FITC-OVA and MHC II were then detected by confocal microscopy.

(B) The developmentally synchronized cell populations were pulsed in suspension culture for the indicated times with FITC-OVA or LY at 2 mg/ml. Flow cytometry was used to quantify tracer internalization. Mean fluorescence intensities representing internalized tracer are plotted against tracer pulse time for each population. Data points represent the average of three experiments and each point represents a minimum of 50,000 events.

Results

Endocytosis Is Rapidly Downregulated during DC Development

DC maturation exhibits three morphologically distinct stages exhibited over a time course of ~20 hr: immature (MHC II enriched in lysosomes), intermediate (MHC II in peripheral non-lysosomal vesicles and on the cell surface), and mature (MHC II on the cell surface) (Pierre et al., 1997; Mellman et al., 1998). Although it has already been demonstrated that endocytosis is downregulated upon maturation of human monocyte derived DCs (Sallusto et al., 1995), we wished to determine if endocytosis was similarly regulated in mouse bone marrow derived DCs, and if so, if it occurred early or late in the developmental sequence. Developmentally synchronized populations of maturing DCs were prepared from mouse bone marrow, exposed to fluorescent endocytic tracers for 5–60 min at 37°C, then examined by fluorescence microscopy and flow cytometry to determine their endocytic capabilities.

As shown in Figure 1A, immature DCs internalized significant amounts of FITC-ovalbumin (FITC-OVA) which was partially localized to MHC II-positive lysosomes within just 30 min. By contrast, in intermediate DCs, relatively little FITC-OVA was taken up, although what little was internalized found its way to perinuclear vesicles, shown to be lysosomes by lamp-1 staining (not shown). In mature DCs, FITC-OVA uptake was not detectable.

Essentially the same results were obtained when the kinetics of FITC-OVA or lucifer yellow (LY) endocytosis were measured quantitatively by flow cytometry (Figure 1B). Uptake of FITC-OVA by immature cells increased continuously for at least 30 min, although LY accumulation appeared to plateau by this time, perhaps reflecting the onset of maturation in the cultures used in this experiment (maturation can be induced simply by physical manipulation of the cultures). Developmentally synchronized cultures of intermediate DCs internalized >5-fold less of these probes over a 30 min time course and, again, uptake of either FITC-OVA or LY was not detected in mature DCs. Thus, the receipt of a maturation stimulus appeared to rapidly inhibit endocytosis, with intermediate DCs already exhibiting a marked decrease in uptake of two fluid phase markers.

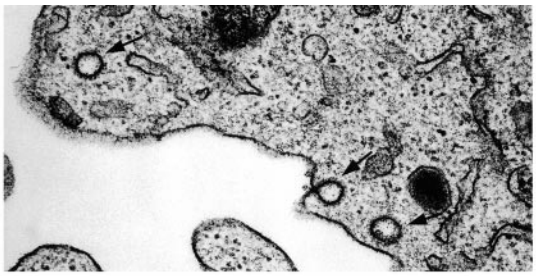
DC Maturation Does Not Decrease Plasma Membrane Clathrin Coated Pits and Vesicles

Having established that maturation induces a rapid inhibition of endocytosis, we next asked if DCs selectively inhibited any particular form of endocytosis. To determine if maturation affected the clathrin coated vesicle pathway, we measured whether maturation decreased plasma membrane coated pits (CPs) and coated vesicles (CVs). As above, synchronized populations of immature and mature DCs were generated (intermediate DCs were not used), fixed, and processed for electron microscopy. Random sections were photographed and subjected to stereological analysis to calculate the number of CPs and CVs per section profile for each population. We normalized these values by calculating the unit plasma membrane length in each sample (Pypaert and Warren, 1992).

Abundant CPs and CVs were easily observed in both immature and mature DCs (Figure 2). Immature DCs exhibited an average of 2.67 ± 0.83 clathrin coated pits and vesicles per section profile as compared to an average of 3.60 ± 0.73 for mature DCs. When normalized to plasma membrane length, there continued to be no significant differences between mature and immature cells (0.0275 μm^{-1} ± 0.013 and 0.018 μm^{-1} ± 0.006, respectively). Thus, DC maturation did not result in a reduction of the number of clathrin coated structures. Mature DCs have previously been shown to internalize the cell surface lectin DEC-205 via clathrin coated pits (Jiang et al., 1995), and will internalize transferrin, a ligand well characterized as entering via coated pits when infected with a transferrin receptor expressing recombinant adenovirus (W. S. G. and I. M., unpublished data).

C. difficile Toxin B, an Inhibitor of Rho GTPases, Inhibits Endocytosis in Immature DC

The ability of immature DCs to internalize large volumes of extracellular fluid appears to reflect a marked capacity for continuous macropinocytosis (Sallusto et al., 1995; Norbury et al., 1997; West et al., 1999). Indeed, the accumulation of mannose receptor ligands such as FITC-OVA was only partially (~35%) inhibited by excess yeast mannan (not shown), suggesting that the bulk of such ligands are internalized in the fluid phase and not by receptor-mediated endocytosis, at least in immature



	clathrin coated pits and vesicles per cell profile	clathrin coated pits and vesicles/ surface length of PM per cell profile
Immature DC	2.67+/- 0.83	.0188 μm^{-1} +/- .006
Mature DC	3.60 +/-.73	.0275 μm^{-1} +/- .013

Figure 2. Dendritic Cell Maturation Does Not Decrease Plasma Membrane Clathrin Coated Pits and Vesicles

Mature and immature DCs were fixed and processed for electron microscopy. The number of clathrin coated pits and vesicles per cell profile and the number of clathrin coated pits and vesicles per surface length of plasma membrane per cell profile were quantified as described in the text. Micrographs (34 micrographs per triplicate pellet) were scored for immature DCs and for mature DCs. Both student t tests and paired t tests demonstrated no statistically significant differences between the values obtained for immature and mature cells. Shown here is a representative micrograph of a mature DC. Clathrin coated pits and vesicles are denoted by arrows.

cells. Like phagocytosis, macropinocytosis is an actin-dependent process regulated by one or more members of the Rho GTPases family (Chen et al., 1996; Caron and Hall, 1998; Hardt et al., 1998). To address the possible involvement of these GTPases in DC endocytosis, we first asked if Toxin B (from *Clostridium difficile*) might block uptake in immature DCs. Toxin B irreversibly inactivates many Rho family GTPases by glucosylation, which blocks their capacity for nucleotide exchange (Aktories, 1997).

As shown in Figure 3, Toxin B greatly inhibited the internalization of three fluorescent tracers in immature DCs. Importantly, Toxin B treatment did not induce maturation since the DCs still exhibited the immature phenotype, as indicated by immunofluorescence and flow cytometry (MHC II molecules found predominantly in lysosomes, not shown). Using LY, FITC-OVA, and FITC-dextran (the latter two also bind to mannose receptor), Toxin B greatly reduced the number and intensity of fluorescent endosomes/lysosomes following a 10 min incubation in each tracer (Figure 3A). These data were quantified by flow cytometry which showed that Toxin B decreased accumulation of the tracers by up to 3.5-fold (Figure 3B).

Although Toxin B reduced the largely fluid phase uptake of LY, FITC-OVA, and FITC-dextran, it did not affect the receptor-mediated endocytosis of FITC-transferrin (Tfn) (Figure 3C). We verified that Tfn uptake was receptor mediated by incubating cells with FITC-Tfn in the presence of a 15-fold excess of unlabeled Tfn, under which conditions flow cytometry revealed no detectable cell-associated FITC-Tfn. Fluorescence microscopy confirmed the internalization of the FITC-Tfn by immature DCs (not shown).

The Toxin B sensitivity of endocytosis in immature DCs suggested that endocytosis was dependent on Rho family GTPase regulated macropinocytosis. Since the activation of Cdc42 and Rac induces macropinocytosis and phagocytosis in a variety of systems, we reasoned these GTPases might be potential sites for regulation of endocytosis in DCs.

S. typhimurium Reactivates Macropinocytosis in Mature DC

While *C. difficile* secretes Toxin B that inactivates Rho GTPases, other microbes secrete proteins that exert the opposite effect, thus stimulating macropinocytosis at sites of bacterial attachment. These factors are secreted directly into the host cell cytoplasm via a specialized, needle-like assembly termed a type III secretion apparatus (Kubori et al., 1998). In the case of *S. typhimurium*, one of its effectors, SopE, activates Rho family GTPases by functioning as a guanine nucleotide exchange factor (GEF) for Cdc42 and Rac1 (Chen et al., 1996; Hardt et al., 1998; Rudolph et al., 1999). We then asked if *S. typhimurium* might reactivate macropinocytosis in mature DCs.

Mature and immature DCs were exposed to wild-type bacteria or to an isogenic mutant *inv*A, which is defective in type III secretion and thus unable to secrete factors such as SopE; *inv*A mutant bacteria have failed to induce macropinocytosis in a wide variety of cultured cells (Galán et al., 1992; Chen et al., 1996). When examined by fluorescence microscopy, immature DCs were found capable of internalizing both wild-type and mutant bacteria (Figure 4A). This result was expected since immature DCs constitutively exhibit macropinocytosis and phagocytosis, and therefore internalize even inert particles. In contrast, when cultures of mature DCs were challenged, only wild-type bacteria were capable of gaining entry (Figure 4A). Thus, only *S. typhimurium* capable of injecting the Cdc42- and Rac1-specific GEF SopE, were able to reactivate macropinocytosis in mature DCs. We quantified our fluorescence microscopy results and found that immature and mature DC internalized wild-type *S. typhimurium* with equal efficiency, however, while almost 100% of immature DC internalized the *inv*A mutant, 0% of mature DC internalized *inv*A (Figure 4B).

We confirmed these results using a well established assay that takes advantage of the fact that internalized bacteria are protected from killing by the antibiotic gentamicin added to the culture media (Galán et al., 1992). Protected, i.e., internalized, bacteria were quantified by plating lysed DCs onto bacterial plates and counting the number of colonies that grew. The results from these experiments clearly showed that wild-type but not *inv*A bacteria were capable of gaining entry into mature DCs (Figure 4C). Mature DCs were markedly less efficient at accumulating *inv*A *S. typhimurium* than mature DCs challenged with wild-type bacteria, or immature DCs challenged with either mutant or wild-type *S. typhimurium.*

Microinjection of Rho GTPases Modulates Endocytosis in Developing DC

The results of the previous experiment strongly imply that wild-type *S. typhimurium* can trigger entry into mature DCs by activating Cdc42 or Rac via SopE. Since

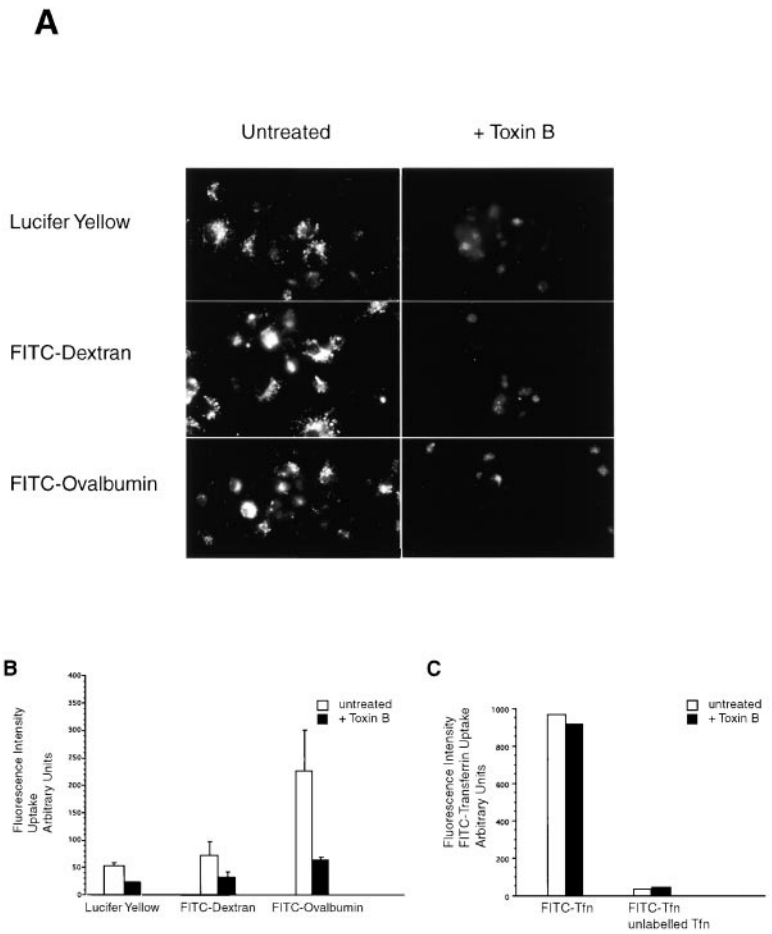


Figure 3. *C. difficile* Toxin B, an Inhibitor of Rho GTPases, Inhibits Endocytosis in Immature Dendritic Cells

After 5 days in culture, immature DCs were pretreated with Toxin B (1 ng/ml) for 18 hr and kept in the presence of Toxin B for the duration of the experiment. Day 6, immature DCs were cluster purified and incubated for 10 min at 37°C with lucifer yellow (LY), FITC-dextran, or FITC-OVA. DCs were then washed, and the internalization of each endocytic tracer assayed by fluorescence microscopy or flow cytometry.

(A) Fluorescence microscopy of immature DCs which were either treated (right panels) or not treated (left panels) with Toxin B, and then exposed to FITC-dextran, LY, or FITC-OVA. Toxin B reduced the uptake of all three endocytic tracers.

(B) Immature DCs treated as in (A) were analyzed quantitatively for FITC-dextran, LY, or FITC-OVA uptake by flow cytometry. Shown are the mean fluorescence intensities for treated versus nontreated samples. Data represents the average of 3 experiments; error bars denote standard error of the mean.

(C) Immature DCs were treated with Toxin B as above and then incubated on ice for 1 hr with FITC-Tfn in the presence or absence of 15-fold excess unlabeled Tfn. The cells were then washed to remove unbound Tfn, warmed to 37°C for 1 hr, and analyzed by flow cytometry. As shown, Toxin B did not affect the uptake of prebound Tfn. FITC-Tfn was not detected in cells incubated with excess unlabeled Tfn, indicating that internalization was due to receptor-mediated uptake. Cells kept at 0°C for 1 hr after ligand binding did not exhibit any cell-associated FITC-Tfn, due to rapid dissociation of the ligand from noninternalized surface receptors. Fluorescence microscopy (not shown) confirmed that cell-associated Tfn at 37°C was intracellular.

these bacteria inject a variety of gene products into their intended hosts, it remained possible that additional factors might play a role. To address whether Rho GTPases directly modulate endocytosis in DCs, we microinjected purified recombinant Cdc42 and Rac1 proteins.

We first attempted to inhibit endocytosis in immature DC by inactivating endogenous Cdc42 or Rac1 by microinjecting their respective dominant-negative mutant forms, Cdc42-N17 and Rac1-N17 (Ridley et al., 1992). As shown in Figure 5A, injection of Cdc42-N17 or Rac1-N17 blocked endocytosis of hen egg lysozyme (HEL) (green) by immature DCs. A similar result was obtained by injecting an N-terminal fragment of the Wiskott-Aldrich syndrome protein (WASP 201–321) corresponding to its Cdc42/Rac1 binding ("CRIB") domain. The WASP 201–321 fragment can be expected to sequester endogenous Cdc42 and Rac1. Neither mock injection nor injection of a constitutively active Cdc42-L61 mutant reduced the ability of immature DCs to internalize HEL, showing that microinjection per se did not reduce endocytosis (Figure 5A). Quantification of these data revealed that cells injected with Cdc42-N17 (n = 554), Rac1-N17 (n = 420), or the N-terminal WASP fragment (n = 312) did not internalize detectable HEL (Figure 5A). Similar results were obtained if FITC-OVA, FITC-dextran, or bacteria were used as endocytic tracers. Thus, inactivation of Cdc42 and Rac1 by injection of dominant negative Cdc42, Rac1, or WASP mutants inhibited endocytosis in immature DCs.

We next asked if we could restore endocytosis in mature DCs by injecting constitutively activated Cdc42 or Rac1. Cells were injected and assayed for endocytosis by a 5 min incubation in HEL to reduce the chances for secondary effects of these proteins. As shown in Figure 5B, both Cdc42-L61 and Rac1-L61 resulted in HEL internalization in mature DCs. Importantly, injection of purified SopE, the *S. typhimurium* Cdc42/Rac1 guanine nucleotide exchange factor, also induced HEL internalization in mature cells. All three proteins reactivated endocytosis in up to 50% of the cells injected, although Cdc42-L61 was most effective (Figure 5B, bottom). In contrast, neither Cdc42 nor GST alone induced endocytosis in mature DCs (Figure 5B); injections of these proteins or activated Cdc42 or Rac1 into immature DCs had no effect on their already active levels of endocytosis. The amounts of uptake observed in the reactivated cells were similar to that observed in immature DCs in a similar 5 min endocytosis assay (not shown).

The ability of both Cdc42-L61 and Rac1-L61 to activate endocytosis in mature DCs suggested that Cdc42 might act via Rac1, or vice versa. To test this possibility, we injected Cdc42-L61 together with dominant-negative Rac1-N17. As shown in Figure 5B, Rac1-N17 was unable

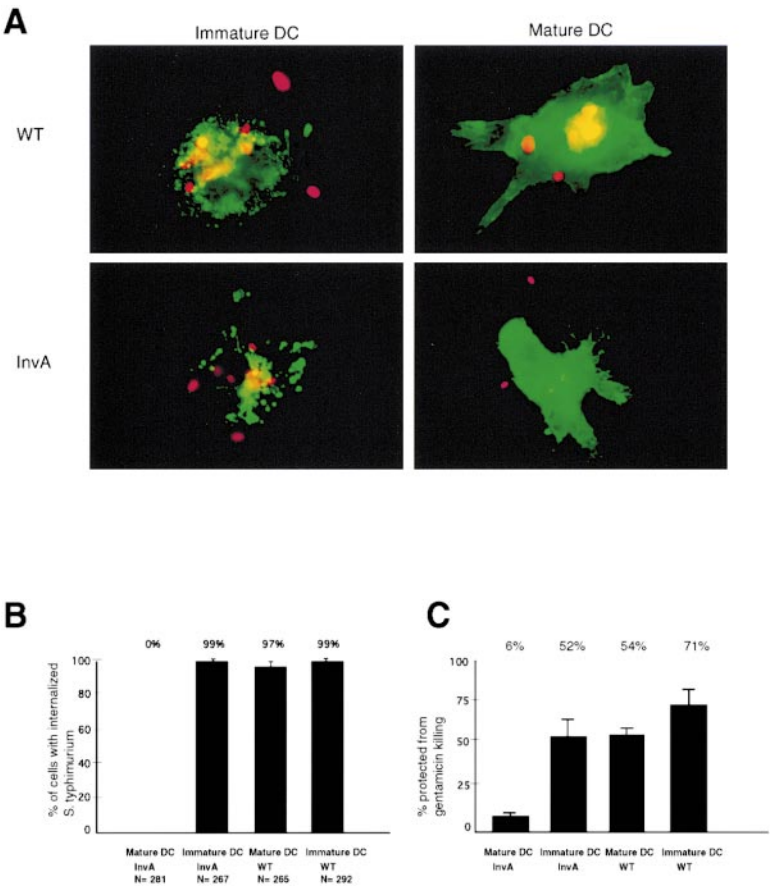


Figure 4. *S. typhimurium* Reactivates Macropinocytosis in Mature Dendritic Cells

(A) Immature (left) and mature (right) DCs were incubated with either wild-type (upper panels) or its isogenic variant mutant *inv*A *S. typhimurium* (lower panels) for 20 min. Cells were then processed for immuno-fluorescence microscopy using anti-class II and anti-Salmonella antibodies. Multiple optical sections using a Zeiss Axiophot microscope were taken to determine if individual bacteria were intracellular. MHC II is stained in green, *S. typhimurium* in red.

(B) Random fields of cells from the above experiment were counted and scored for internalization. Mean percentages of internalization were determined for each condition. Error bars denote standard error of the mean.

(C) Internalized *S. typhimurium* were quantified using a gentamicin protection assay. Immature and mature DCs were gently washed in antibiotic-free media and infected with either wild-type or its isogenic variant mutant *inv*A *S. typhimurium* at an MOI of 10. Following infection, DCs were incubated with gentamicin, washed extensively, lysed in Triton X-100, and cell lysates were plated on agar with antibiotics. The percentage of bacteria that were protected from gentamicin killing is shown. Error bars denote standard error of the mean from at least three separate determinations.

to block Cdc41-L61-induced HEL uptake. Similarly, Rac1-N17 failed to block endocytosis induced by the nulceotide exchange factor SopE (Figure 5B), suggesting that activation of endogenous Cdc42 in mature DCs also elicited HEL uptake in a Rac1-independent fashion. On the other hand, the ability of the active Rac1-L61 mutant to induce endocytosis was inhibited by coinjection of Cdc42-N17, raising the possibility that the action of Rac1 on HEL internalization was indirect, requiring activatable Cdc42. As before, identical results were obtained regardless of whether HEL, FITC-OVA, FITC-dextran, or bacteria were used as endocytic tracers.

Together, these data demonstrate that Cdc42 or Rac1 could itself control the endocytic potential of immature and mature DCs, however, the coinjection experiments suggest that Cdc42 may be the more likely effector protein. Since active Cdc42 was effective at reactivating macropinocytosis in otherwise quiescent mature DCs, these findings also suggest that the components required for macropinocytosis downstream from Cdc42 are relatively unaffected by DC maturation.

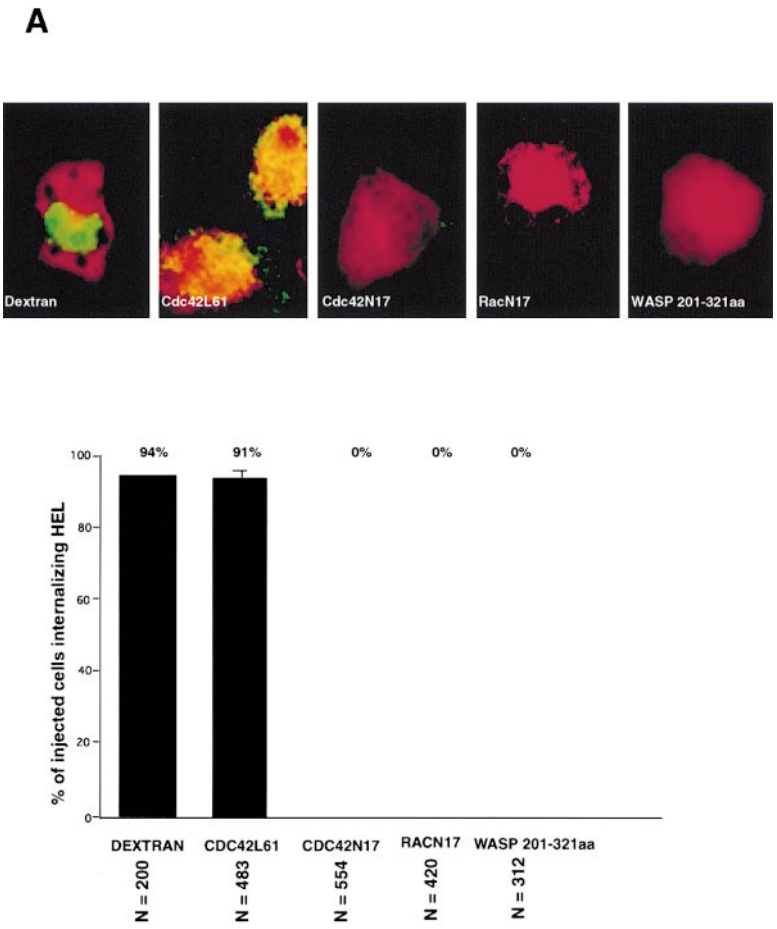
Dendritic Cells Regulate Endocytosis by Regulating the Activation of Cdc42

The fact that microinjection of mutant alleles of Cdc42 and Rac1 reversed the distinctive endocytic capacities of immature and mature DCs suggested that this mechanism might actually be used to control endocytosis during maturation. Therefore, we next asked if there were differences in the levels of activated (i.e., GTP-bound) Cdc42 and Rac1 between immature and mature DCs. In addition, we monitored the activation state of Rac2, a closely related isoform expressed by many immune cells (Didsbury et al., 1989).

We determined the levels of GTP-bound Cdc42, Rac1, and Rac2 in immature and mature DCs by assaying the ability of the endogenous GTPases to bind p21 activated kinase (PAK). This well established assay is based on the fact that PAK will only bind Cdc42 or Rac in their activated, GTP-bound forms (Taylor and Shalloway, 1996; Bagrodia et al., 1998). DC lysates were incubated with GST-PAK coupled to Sepharose beads and the resulting eluates were probed for Cdc42 or Rac by Western blot. The total levels of Cdc42, Rac1, and Rac2 were also measured in total cell lysates of both immature and mature DCs.

Although immature and mature DCs expressed similar amounts of Cdc42 protein (Figure 6A, lanes 1 and 2), only immature DCs contained detectable Cdc42-GTP bound to PAK-Sepharose beads (Figure 6A). Cdc42-GTP could not be detected in lysates of mature DCs. Moreover, when mature and immature DC lysates were coincubated with the GST-PAK beads, activated Cdc42 was detected in the mixed sample at a level comparable to that of immature DCs, indicating that the mature DC lysates did not contain an activity which hydrolyzed or destroyed Cdc42-GTP (not shown).

This pattern of regulation was not observed for either Rac1 or Rac2. Although comparable levels of Rac protein were detected in both immature and mature DCs, similar amounts of GTP-bound Rac1 and Rac2 also bound to GST-PAK beads (Figures 6B and 6C). Thus, unlike Cdc42, neither Rac1 nor Rac2 activation was developmentally regulated, suggesting that the ability of



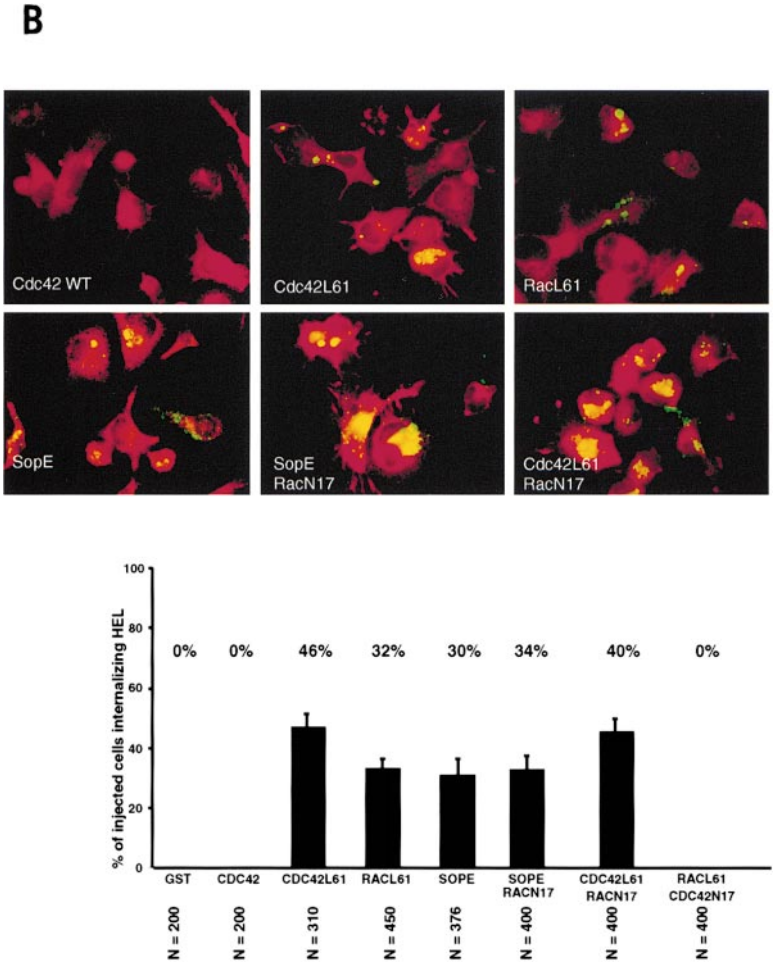


Figure 5. Microinjection of Rho GTPases Modulates Endocytosis in Developing Dendritic Cells

DCs were microinjected with purified recombinant Cdc42 and Rac1 proteins (with or without GST-fusion). Injected cells were identified by coinjection of Texas red dextran (10,000 MW) or by an anti-GST antibody. After 20 min, cells were pulsed with HEL (green) for 5 min and processed for immuno-fluorescence microscopy.

(A) Immature DCs were injected with the following: Texas red dextran, Cdc42L61-GST, Cdc42N17, Rac1N17, and WASP 201–321 aa. The number of injected cells (red) that internalized HEL (green) was quantified visually and plotted as a percentage of the total number of injected cells. The total number of cells counted is shown from a total of three separate experiments.

(B) Mature DCs were injected with the following: GST (not shown), Cdc42 WT-GST, Cdc42L61-GST, Rac1L61-GST, SopE-GST, SopE-GST/Rac1N17-GST, Cdc42L61-GST/Rac1N17-GST, and Rac1L61-GST/Cdc42N7-GST (not shown). The number of injected cells (red) that internalized HEL (green) was quantified visually and plotted as a percentage of the total number of injected cells. The total number of cells counted is shown from a total of three separate experiments.

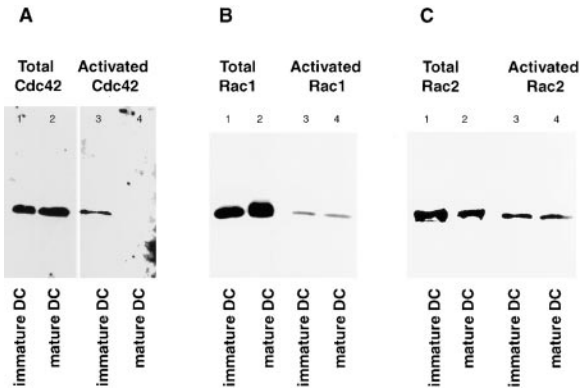


Figure 6. Dendritic Cells Regulate Endocytosis by Regulating the Activation of Cdc42

To observe the total endogenous levels of Cdc42, Rac1, and Rac2, lysates of immature or mature DCs ($5x10^5$ cells) were probed by Western blot using antibodies to Cdc42 (lanes 1 and 2 in [A]), Rac1 (lanes 1 and 2 in [B]), and Rac2 (lanes 1 and 2 in [C]). The active (GTP-bound) forms of the same GTPases were examined in lysates of immature or mature DCs ($1.5x10^8$ cells) by the ability to bind GST-PAK coupled to glutathione-Sepharose beads in vitro: Cdc42 (lanes 3 and 4 in [A]), Rac1 (lanes 3 and 4 in [B]), and Rac2 (lanes 3 and 4 in [C]).

DCs to downregulate endocytosis upon maturation reflects the selective regulation of Cdc42-GTP.

Discussion

Our results strongly suggest that the ability of DCs to dramatically downregulate their capacity for endocytosis upon maturation reflects, at least in part, an ability to selectively regulate levels of activated Cdc42. Four distinct lines of evidence support this general conclusion: 1) the ability of Toxin B, a specific inhibitor of Cdc42 and Rac, to inhibit endocytosis in immature DCs; 2) the ability of *S. typhimurium*, a bacterium which induces its own uptake by activating Cdc42 in its intended host, to enter mature DCs; 3) the finding that microinjection of active or inactive mutants of Cdc42 (and Rac1) either activated or abolished endocytosis in mature and immature DCs, respectively; and most importantly, 4) the finding that endogenous levels of Cdc42-GTP are markedly reduced in mature versus immature DCs. Thus, DCs would appear to control endocytosis by making use of Cdc42 as a regulatory switch controlling an actin-dependent event. Perhaps not coincidentally, the same switch is targeted for activation by pathogens such as *S. typhimurium* and *Shigella flexnerii*, whose ability to trigger endocytosis is for them a matter of life and death (Galán, 1999; Nhieu and Sansonetti, 1999).

Injection of Cdc42-L61 was effective at reactivating endocytosis in ~50% of injected mature DCs, indicating that the machinery necessary for macropinocytosis and phagocytosis downstream of Cdc42 remains largely intact after maturation. Although other factors probably exist which also regulate endocytosis in DCs, it is likely that the inability of the activated mutant to completely restore endocytosis reflected its inability to reproduce the normal Cdc42 cycle. Certainly, wild-type *S. typhimurium* induced entry into almost all of the mature DCs with which it came in contact, presumably by activating endogenous Cdc42-GDP.

It was striking that maturation caused a selective decrease in levels of Cdc42-GTP and not of Rac1- or Rac2-GTP. The functions of these Rho family GTPases can be difficult to distinguish, particularly when assaying activities such as membrane ruffling or phagocytosis following microinjection or transfection (Hall, 1998). Indeed, based on our own microinjection experiments, Cdc42 was at best only slightly more effective than Rac1 in its ability to activate or inactivate endocytosis in mature or immature DCs, respectively. This result might reflect redundancy in function between endogenous Cdc42 and overexpressed Rac1, or that Rac1 acts downstream from Cdc42. However, the observation that Rac1-N17 failed to block the activation of endocytosis by Cdc42-L61 in mature DCs suggested that Cdc42 function was not dependent on Rac1. Dependence on Rac2 also seemed unlikely given that DCs from Rac2 knockout mice did not exhibit alterations in endocytosis (W. S. G. and I. M., unpublished data).

The precise mechanism by which Cdc42 acts to regulate endocytosis in DCs or other cells remains to be determined. However, Cdc42 does not act by simply controlling membrane ruffling. Using video microscopy, we have found that both immature and mature DCs appeared to exhibit comparable ruffling activity while only immature cells were capable of simultaneously accumulating extracellular FITC-dextran (W. S. G. and I. M., unpublished data). Cdc42 exists in the cytosol in the GDP form bound to Rho-GDI (guanine nucleotide dissociation inhibitor) and is recruited to membranes where its GDP is exchanged for GTP due to the action of one or more GEFs. Although much of the membrane-associated Cdc42 in resting cells is associated with the Golgi complex (Erickson et al., 1996; Kroschewski et al., 1999), a variety of signals can cause its redistribution to peripheral membranes often accompanied by a marked polymerization of actin (Hall, 1998). Cdc42-GTP binds to the linker protein WASP (or to the ubiquitously expressed N-WASP) which in turn binds the Arp 2/3 complex triggering actin filament formation (Ma et al., 1998; Abdul-Manan et al., 1999; Mott et al., 1999). This series of events could provide the force required to form membrane extensions required for macropinosome or phagosome formation. It also might promote the actin-dependent motility of intracellular vesicles which are recruited at areas of high membrane activity (Bajno et al., 2000; Taunton et al., 2000). Cdc42 might even propel newly formed macropinosomes to the interior of the cell (Merrifield et al., 1999). In this context, it is worth noting that DCs from WASP-deficient mice fail to show abnormalities in endocytosis or MHC II trafficking (W. S. G. and I. M., unpublished data). However, DCs also express N-WASP, which can be presumed to provide a compensatory function.

Regardless of the role played by Cdc42, a key unknown is the mechanism targeted by developing DCs to regulate its activation. One attractive possibility is that the regulation is exerted at the level of GEFs, which facilitate exchange of GDP for GTP. The exchange rate influences the biological output of a small GTPase, e.g., active macropinocytosis when there is a high rate of exchange or the cessation of macropinocytosis when exchange rates decrease. DC maturation may turn off a signal required for the activation or localization of a Cdc42 GEF, altering the rate of GDP–GTP exchange. Conceivably, such signals could be generated by receptors that detect maturation stimuli (e.g., lipopolysaccharide). An alternative might be that maturation induces a

factor which sequesters or inactivates Cdc42, although mixing experiments show that mature DCs do not contain an activity which reduces the level of Cdc42-GTP in lysates from immature DCs. However, it remains possible that a downstream effector of Cdc42 might be regulated such that it binds Cdc42-GTP without triggering GTP hydrolysis or interfering with the recycling of Cdc42-GDP. Indeed, other DC populations which differ in lineage or function may make use of distinct strategies to regulate endocytosis. Splenic DCs, for example, may also rely on mechanisms downstream to that provided by Cdc42 (C. Watts, personal communication).

Given that *S. typhimurium* acts by regulating Cdc42 and Rac activity at the level of nucleotide exchange (by injecting the GEF SopE), the idea of a developmentally regulated exchanger is, we feel, the most appealing. A variety of endogenous GEFs have been identified that are specific for the Rho GTPases. In yeast, the GEF Cdc24 is selective for Cdc42, with the closest mammalian homolog of Cdc24 being the proto-oncogene Dbl (Cerione and Zheng, 1996; Whitehead et al., 1997; Stam and Collard, 1999). The expression of some of these Dbl GEF family members is even restricted to lymphoid cells. Given that nucleotide exchange assays in vitro can be somewhat promiscuous in terms of their GTPase specificity, it remains to be determined which, if any, of these GEFs represent the best candidate in DCs.

Although DC maturation provides one of the most dramatic examples of endocytosis downregulation yet observed, what is the significance of this event for DC function in antigen presentation and the immune response? One function might be to ensure the longevity of peptide–MHC II complexes at the cell surface (Cella et al., 1997b). In addition, downregulation of endocytosis may be a factor in determining the spectrum of antigens presented to T cells. This, in turn, may help determine whether DCs help induce immunity to foreign antigens or tolerance to self antigens. We have recently found that actual loading onto MHC class II molecules of immunogenic peptides derived from these antigens is also tightly regulated: peptide loading can only occur after the receipt of a maturational stimulus (Inaba et al., 2000; Turley et al., 2000). By synchronizing the cessation of endocytosis with the activation of peptide loading onto MHC II molecules, DCs limit their presentation activity to those antigens encountered prior to or concomitant with an inflammatory stimulus. In any event, it is clear that the mechanism of DC maturation demonstrates the role of Cdc42 as an important element in the normal control of endocytosis.

Experimental Procedures

Mouse DC Culture

Male BDF1, C3H, C3H/HeJ, 4–6 week-old mice were purchased from Jackson Laboratories or (if available) from Charles River Laboratories and bone marrow–derived DCs were cultured as previously described (Inaba et al., 1992, 2000). Recombinant mouse GM-CSF was produced as culture supernatant from J558L cells transfected with the mouse GM-CSF cDNA (a gift of Dr. D. Gray, London, UK). Cells were cultured in RPM1, 5% FCS, 50μm β-mercaptoethanol, and recombinant mouse GM-CSF. Nonadherent cells were removed carefully and fresh media was added every two days. At day 6, DC clusters, which were of the "immature phenotype", were dislodged. For intermediate phenotype DC, dislodged DC clusters were disaggregated and replated for 8–12 hr. For mature phenotype DC, dislodged DC clusters were disaggregated and replated with 100 ng/ml LPS from *S. typhimurium* (Sigma) for 36–72 hr. DC clusters were further purified by unit gravity sedimentation on 6 ml 50% FCS columns (Inaba et al., 1992).

Antibodies and Immunocytochemistry

Immuno-fluorescence patterns were visualized with fluorescent or confocal microscopy as described (Pierre et al., 1997). Mouse I-A was detected using an affinity purified rabbit polyclonal antibody, "Rivoli", directed against the conserved MHC II I-A β chain cytoplasmic tail peptide (KGPRGPPPAGLLQ); mouse Lamp-1 using the rat anti-mouse monoclonal (Pierre and Mellman, 1998); mouse MHC II using TIB120, a rat monoclonal; and GST fusion proteins using a rabbit polyclonal anti-GST antibody (Sigma). A rabbit polyclonal antisera against sero-group B *Salmonella* was used to detect SB136 and SB300 *S. typhimurium*. HEL was detected using 1B12, a mouse monoclonal (provided by Dr. P. Allen, Washington University, St. Louis, MO).

Endocytosis Assays

Flow Cytometry Assay

Endocytic tracer was either added directly to DC culture dishes or to DC populations harvested in polypropylene tubes and then incubated for the prescribed time. For immature DC fed in culture dishes, the DC clusters were cluster purified on ice as described (Inaba et al., 1992). After the cells were pulsed with endocytic tracer, cells were transferred to pre-cooled tubes and washed 4X with PBS 2% FCS in the cold. (Sallusto et al., 1995) Following the washes, cells were stained with either phyco-erythrin anti -MHC II (anti I-Ak, anti-I-Ab,d) or phyco-erythrin anti-CD86 antibody (Pharmingen), washed, and then evaluated on a FACSCalibur. MHC II and CD86 staining allowed for further identification and staging of the DC populations. Uptake of the fluoresceinated endocytic tracer was measured in the FL1 channel. Background control cells were pulsed on ice with endocytic tracer, washed, and processed as above.

Immuno-fluorescence Assay

For time course experiments assessing fluid phase endocytosis, DC were harvested, plated on Alcian Blue coated coverslips, and inverted onto a 40 μl droplet of endocytic tracer in RPMI 5% FCS at 37°C. Coverslips were washed in cold PBS with FCS 2% 4×, cold PBS 2×, and then fixed in 4% PFA. For receptor mediated endocytosis experiments, DC were harvested, cooled, and incubated on ice with the ligand for up to 2 hr. Cells were then washed in the cold with PBS 2% FCS 4X, plated on Alcian Blue coverslips in the absence of serum for 20 min at 37°C, and then fixed in 4% PFA. Concentrations for endocytic tracers were as follows: Lucifer Yellow potassium salt 2.5 mg/ml, FITC-ovalbumin 2mg/ml, FITC-bovine serum albumin 2 mg/ml, FITC-dextran 40,000 molecular weight lysine fixable 2 mg/ml (Molecular Probes), HEL 2 mg/ml, FITC-Tfn 2 mg/ml, holo-Tfn 30 mg/ml (Sigma), *Salmonella typhimurium* SB300 wt streptomycin resistant 10–100/cell, *S. typhimurium* SB136 invA kanamycin resistant 10–100/cell.

Electron Microscopy

Sample Preparation

DC were harvested from their culture dishes and an equal volume of 2X fixative (5% glutaraldehyde, 200mM Na cacodylate, pH 7.4) was added to the cells in suspension. The suspension was gently inverted and incubated for 15 min. Cells were spun at 200 × g for 5 min, fixative was aspirated, cells were resuspended in 1× fixative, transferred to a 1.5 ml tube, and incubated for 30 min. Cells were spun in an microfuge at 3000 rpm for 1 min, resuspended in 1M sodium phosphate (pH 7.0), and incubated for 10 min. Cells were spun as before, washed, pelleted, treated with 2% OsO_4 for 1 hr, dehydrated using a graded ethanol series and acetone, and pelleted before embedding in Epon for sectioning. Three pellets of immature DC and mature DC were prepared and quantification was performed on sections from the triplicate pellets.

Quantification

Thirty-four micrographs were taken randomly from each triplicate sample of immature and mature cells. Clathrin coated (CC) vesicles were scored according to the following criteria: presence of a structure with a fuzzy coat, ~100 nm in diameter, and within one vesicle diameter of the plasma membrane (PM). CC pits were scored based on the presence of the characteristic CC on the PM. The number

of CC pits and vesicles per unit of PM was calculated as described in Pypaert and Warren (1992). Briefly, the number of PM intersections with the horizontal lines of a 1 cm × 1 cm grid were counted (I). The exact magnification of the negatives was calculated by calibrating the EM with a special replica plate and then the distance between the grid lines at the negative magnification was obtained (D). The relative PM length is 2I/D.

Standard deviations were calculated for the mean number of CC pits and vesicles per sample, the mean PM length, and the mean number of CC pits and vesicles per mean PM length. Two sample t tests and paired t tests were performed on the above data from immature and mature cells.

Gentamicin Protection Assay

Wild-type streptomycin resistant *S. typhimurium* strain SB300 and its isogenic invA mutant strain SB136 were grown under conditions that stimulate the expression of the invasion-associated type III secretion system (Galán et al., 1992; Chen et al., 1996). Day 6 cultures were gently washed with antibiotic free media 3 times and then immature DC clusters were purified. Mature DCs were washed with antibiotic free media three times. DC populations were infected with either wild-type or invA *S. typhimurium* at an MOI of 10 per cell for 90 min. Cells were harvested and then washed with media. Gentamicin (10 μg/ml) was added for 90 min. Cells were washed three times with media and then lysed in 1% Triton X-100 in PBS. Serial dilutions of cell lysates were made in PBS and lysates were plated on LB plates with the appropriate antibiotic. Plates were incubated at 37°C for 24 hr and then colonies were counted.

Toxin B Treatment

Toxin B isolated from *Clostridium difficile* was a gift of Patrice Bouquet. Toxin B (final concentration of 1 ng/ml) was added to DC cultures on day 5. Cells were pretreated for 18–24 hr and then immature DCs were harvested and their endocytic activity was assessed using both flow cytometry and immuno-fluorescence assays.

Microinjection

DCs were plated on poly-L-lysine coverslips for 20 min in the absence of FCS, nonattached cells were aspirated, and RPM1 containing 20 mM HEPES and 5% FCS was added back to the dishes containing the coverslips. Where noted, protein or peptides (0.3 mg/ml) were mixed with Texas red dextran MW 10,000 (10 mg/ml final concentration) (Sigma) and injected into the cytoplasm of ~500 cells over a period of <20 min using an Eppendorf injection system mounted on a Zeiss Axiovert microscope with a 37°C heated stage. The endocytic capacity of injected and control injected cells was assessed by inverting injected coverslips onto a prewarmed droplet of endocytic tracer for 5 min, washing the coverslip with PBS 2% FCS 4×, PBS 2×, and fixing with PFA 4%. Cells were then stained for class II as described (Pierre et al., 1997).

Protein Purification

PGEX-PAK binding domain (PBD) -GST, PGEX-Cdc42N17-GST, PGEX-Cdc42L61-GST, PGEX-Rac1N17-GST, PGEXRac1L61, PGEX-N-WASp201–321, and PGEX-SopE-GST were purified as described (Guan and Dixon, 1991). Bacterial cell lysates were subsequently incubated with gluthathione-Sepharose and washed. When indicated, GST was cleaved with a thrombin cleavage capture kit (Novagen). We have injected GST fusions, thrombin cleaved GST fusions, and commercially purified preparations (Cytoskeleton Company) and observed no differences in their effects on endocytosis.

PAK Assay

Affinity precipitation with GST-PAK Cdc42/Rac binding domain (GST-PBD) was as described (Bagrodia et al. 1998). Eighty microliters of freshly prepared GST-PBD glutathione beads was added to 0.5 ml of the cell lysate and rocked at 4°C for 25 min. Recombinant constitutively active mutant proteins were used as a positive control and recombinant dominant-negative mutant proteins were used as a negative control in the PAK assay. The beads were pelleted in a microfuge and washed 3 times with lysis buffer. SDS sample buffer was added to the beads, samples were boiled for 3 min, and then loaded on 12.5% acrylamide gels. Gels were transferred to nitrocellulose and probed with a mouse monoclonal anti-Cdc42 (Transduction Laboratories), with a mouse monoclonal anti-Rac1 (Transduction Laboratories), or with a rabbit polyclonal anti-Rac2 (a gift of Gary Bokoch, Scripps Institute, LaJolla, CA) and horseradish peroxidase-coupled secondary Abs (Jackson ImmunoResearch). Blots were visualized using West Pico Chemiluminescent Reagent (Pierce).

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References

Abdul-Manan, N., Aghazadeh, B., Liu, G.A., Majumdar, A., Ouerfelli, O., Siminovitch, K.A., and Rosen, M.K. (1999). Structure of Cdc42 in complex with the GTPase-binding domain of the 'Wiskott-Aldrich syndrome' protein. Nature *399*, 379–383.

Aktories, K. (1997). Bacterial toxins that target Rho proteins. J. Clin. Invest. *99*, 827–829.

Austyn, J.M. (1996). New insights into the mobilization and phagocytic activity of dendritic cells. J. Exp. Med. *183*, 1287–1292.

Bagrodia, S., Taylor, S., Jordon, K.A., Van Aelst, L., and Cerione, R.A. (1998). A Novel Regulator of p21-activated Kinases. J. Biol. Chem. *273*, 23633–23636.

Bajno, L., Peng, X.R., Schreiber, A.D., Moore, H.P., Trimble, W.S., and Grinstein, S. (2000). Focal exocytosis of VAMP3-containing vesicles at sites of phagosome formation. J. Cell Biol. *149*, 697–706.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245–252.

Caron, E., and Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science *282*, 1717–1721.

Cella, M., Sallusto, F., and Lanzavecchia, A. (1997a). Origin, maturation and antigen presenting function of dendritic cells. Curr. Opin. Immunol. *9*, 10–16.

Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997b). Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature *388*, 782–787.

Cerione, R.A., and Zheng, Y. (1996). The Dbl family of oncogenes. Curr. Opin. Cell Biol. *8*, 216–222.

Chen, L.M., Hobbie, S., and Galán, J.E. (1996). Requirement of CDC42 for Salmonella-induced cytoskeletal and nuclear responses. Science *274*, 2115–2118.

Cox, D., Chang, P., Zhang, Q., Reddy, P.G., Bokoch, G.M., and Greenberg, S. (1997). Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. J. Exp. Med. *186*, 1487–1494.

Didsbury, J., Weber, R.F., Bokoch, G.M., Evans, T., and Snyderman, R. (1989). rac, a novel ras-related family of proteins that are botulinum toxin substrates. J. Biol. Chem. *264*, 16378–16382.

Driessen, C., Bryant, R.A., Lennon-Dumenil, A.M., Villadangos, J.A., Bryant, P.W., Shi, G.P., Chapman, H.A., and Ploegh, H.L. (1999). Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells. J. Cell Biol. *147*, 775–790.

Erickson, J.W., Zhang, C., Kahn, R.A., Evans, T., and Cerione, R.A.

(1996). Mammalian Cdc42 is a brefeldin A-sensitive component of the Golgi apparatus. J. Biol. Chem. *271*, 26850–26854.

Galán, J.E. (1999). Interaction of Salmonella with host cells through the centisome 63 type III secretion system. Curr. Opin. Microbiol. *2*, 46–50.

Galán, J.E., Ginocchio, C., and Costeas, P. (1992). Molecular and functional characterization of the Salmonella invasion gene invA: homology of InvA to members of a new protein family. J. Bacteriol. *174*, 4338–4349.

Guan, K.L., and Dixon, J.E. (1991). Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. *192*, 262–267.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509–514.

Hardt, W.D., Chen, L.M., Schuebel, K.E., Bustelo, X.R., and Galán, J.E. (1998). S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell *93*, 815–826.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. *176*, 1693–1702.

Inaba, K., Inaba, M., Naito, M., and Steinman, R.M. (1993). Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. J. Exp. Med. *178*, 479–488.

Inaba, K., Turley, S., Yamaide, F., Iyoda, T., Mahnke, K., Inaba, M., Pack, M., Subklewe, M., Sauter, B., Sheff, D., et al. (1998). Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. J. Exp. Med. *188*, 2163–2173.

Inaba, K., Turley, S.J., Iyoda, T., Yamaide, F., Shimoyama, S., Reis e Souza, C., Germain, R., Mellman, I., and Steinman, R.M. (2000). The Formation of immunogenic MHC class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. J. Exp. Med. *191*, 927–936.

Jiang, W., Swiggard, W.J., Heufler, C., Peng, M., Mirza, A., Steinman, R.M., and Nussenzweig, M.C. (1995). The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature *375*, 151–155.

Kroschewski, R., Hall, A., and Mellman, I. (1999). Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. Nat. Cell Biol. *1*, 8–13.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galán, J.E., and Aizawa, S.I. (1998). Supramolecular structure of the Salmonella typhimurium type III protein secretion system. Science *280*, 602–605.

Li, G., D'Souza-Schorey, C., Barbieri, M.A., Cooper J.A., and Stahl, P.D. (1997). Uncoupling of membrane ruffling and pinocytosis during Ras signal transduction. J. Biol. Chem. *272*, 10337–10340.

Ma, L., Rohatgi, R., and Kirschner, M.W. (1998). The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. Proc. Natl. Acad. Sci. USA *95*, 15362–15367.

Massol, P., Montcourrier, P., Guillemot, J.C., and Chavrier, P. (1998). Fc receptor-mediated phagocytosis requires CDC42 and Rac1. EMBO J. *17*, 6219–6229.

Mellman, I., Turley, S.J., and Steinman, R.M. (1998). Antigen processing for amateurs and professionals. Trends Cell Biol. *8*, 231–237.

Merrifield, C.J., Moss, S.E., Ballestrem, C., Imhof, B.A., Giese, G., Wunderlich, I., and Almers, W. (1999). Endocytic vesicles move at the tips of actin tails in cultured mast cells. Nat. Cell. Biol. *1*, 72–74.

Mott, H.R., Owen, D., Nietlispach, D., Lowe, P.N., Manser, E., Lim, L., and Laue, E.D. (1999). Structure of the small G protein Cdc42 bound to the GTPase-binding domain of ACK. Nature *399*, 384–388.

Nhieu, G.T., and Sansonetti, P.J. (1999). Mechanism of Shigella entry into epithelial cells. Curr. Opin. Microbiol. *2*, 51–55.

Norbury, C.C., Chambers, B.J., Prescott, A.R., Ljunggren, H.G., and Watts, C. (1997). Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. Eur. J. Immunol. *27*, 280–288.

Pierre, P., and Mellman, I. (1998). Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. Cell *93*, 1135–1145.

Pierre, P., Turley, S.J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R.M., and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. Nature *388*, 787–792.

Pypaert, M., and Warren, G. (1992). Morphological studies of formation of coated pits and coated vesicles in broken cells. Methods Enzymol. *219*, 212–222.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell *70*, 401–410.

Rudolph, M.G., Weise, C., Mirold, S., Hillenbrand, B., Bader, B., Wittinghofer, A., and Hardt, W.D. (1999). Biochemical analysis of SopE from Salmonella typhimurium, a highly efficient guanosine nucleotide exchange factor for RhoGTPases. J. Biol. Chem. *274*, 30501–30509.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: down regulation by cytokines and bacterial products. J. Exp. Med. *182*, 389–400.

Stam, J.C., and Collard, J.G. (1999). The DH protein family, exchange factors for Rho-like GTPases. Prog. Mol. Subcell. Biol. *22*, 51–83.

Taunton, J., Rowning, B.A., Coughlin, M.L., Wu, M., Moon, R.T., Mitchison, T.J., and Larabell, C.A. (2000). Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. J. Cell Biol. *148*, 519–530.

Taylor, S.J., and Shalloway, D. (1996). Cell cycle-dependent activation of Ras. Curr. Biol. *6*, 1621–1627.

Turley, S.J., Inaba, K., Garrett, W.S., Ebersold, M., Unterhnaehrer, J., Steinman, R.M., and Mellman, I. (2000). Transport of peptide-MHC class II in developing dendritic cells. Science *288*, 522–527.

Watarai, M., Kamata, Y., Kozaki, S., and Sasakawa, C. (1997). Rho, a small GTP-binding protein, is essential for Shigella invasion of epithelial cells. J. Exp. Med. *185*, 281–292.

West, M.A., Antoniou, A.N., Prescott, A.R., Azuma, T., Kwiatkowski, D.J., and Watts, C. (1999). Membrane ruffling, macropinocytosis and antigen presentation in the absence of gelsolin in murine dendritic cells. Eur. J. Immunol. *29*, 3450–3455.

Whitehead, I.P., Campbell, S., and Rossman, K.L. and Der, C.J. (1997). Dbl family proteins. Biochim. Biophys. Acta. *1332*, F1–23.