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Small Rho GTPases Regulate Antigen Presentation in Dendritic Cells

Galina V. Shurin,* Irina L. Tourkova,* Gurkamal S. Chatta,‡ Gudula Schmidt,§ Sheng Wei,¶ Julie Y. Djeu,¶ and Michael R. Shurin2*†

Dendritic cells (DC) perform an essential role in the initiation of innate and adaptive immunity. They are major contributors to host immunity against infection and malignancy. DC are a heterogeneous group of cells derived from both myeloid and lymphoid precursors, which populate peripheral tissues and lymphoid organs. Their main functions include the following: 1) uptake and processing of different antigenic molecules, 2) migration from peripheral tissues to lymphoid organs, 3) Ag presentation in an MHC class I- and class II-restricted manner, and 4) production of cytokines and expression of costimulatory molecules critical for efficient activation of T cells (1, 2).

DC exist in two functionally and phenotypically distinct states, immature and mature (3). Immature DC are widely distributed throughout the body and occupy sentinel positions in many non-lymphoid tissues. They constantly sample their environment for Ags by phagocytosis, macropinocytosis, and pinocytosis. Immature DC express relatively low levels of MHC class I, class II, and costimulatory molecules (4, 5). After engulfing Ags and activation by proinflammatory cytokines, immature DC differentiate into mature DC, which have a reduced potential for Ag uptake but have a high capacity for Ag presentation and T cell stimulation (3). This transition is accompanied by dramatic cytoplasmic reorganization, characterized by a redistribution of MHC class II from intracellular compartments to the plasma membrane and up-regulation of surface costimulatory molecules (CD80, CD86), CD40, MHC class I, and T cell adhesion molecules (e.g., CD48 and CD58). DC also remodel their profile of chemokine receptors that facilitate migration and homing to lymphoid organs (3). Finally, the cells also extend long dendritic processes that further increase opportunities for T cell capture and interaction. All of the above changes are crucial for DC function, and depend on regulation of actin assembly, which in turn is mediated by the Rho family of GTPases, i.e., Rho, Cdc42, and Rac (5–9).

The Rho GTPases form a subgroup of the Ras superfamily of 20- to 30-kDa GTP-binding proteins that have been shown to regulate a wide spectrum of cellular function (10, 11). The mammalian Rho-like GTPases comprise >20 distinct proteins, including RhoA, -B, -C, -D, and -E; Rac1 and -2, RacC, Cdc42, and TC10. Among all Rho GTPases, Rac1 (Ras-related C3 botulinum toxin substrate 1), Cdc42 (cell division cycle 42), and RhoA (Ras homologous member A) have been studied most extensively. Until recently, members of the Rho subfamily were believed to be involved primarily in the regulation of cytoskeletal organization in response to extracellular growth factors. However, results from a number of laboratories over the past few years have revealed that Rho GTPases play a crucial role in diverse cellular events such as membrane trafficking, transcriptional regulation, cell growth control, endocytosis, differentiation, and apoptosis (12, 13). Activity of Rho GTPases is regulated by signals originating from different classes of surface receptors including G protein-coupled receptors, tyrosine kinase receptors, cytokine receptors, and adhesion receptors (14). Like all members of the Ras superfamily, the Rho GTPases function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state (15). The ratio of the two forms is regulated by the opposing...

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3 Abbreviations used in this paper: DC, dendritic cell; GDI, guanine nucleotide dissociation inhibitor; dn, dominant negative; ca, constitutively active; VV, vaccinia virus; EGFP, enhanced GFP; ToxB, toxin B; CNF1, cytotoxic necrotizing factor 1; PAK-1, p21-activated kinase-1; CRIB, Cdc42/Rac interactive binding; PBD, p21 binding domain; Rhotekin RBD, Rhotekin Rho binding domain; HPF, high power field; WASP, Wiskott-Aldrich syndrome protein.
effect of guanine nucleotide exchange factors, which enhance the exchange of bound GDP for GTP, and the GTPase-activating proteins, which increase the intrinsic rate of hydrolysis of bound GTP. In addition, the Rho-like GTPases are regulated further by guanine nucleotide dissociation inhibitors (GDIs), which can inhibit both the exchange of GTP and the hydrolysis of bound GTP (16). In addition to being an inhibitor of nucleotide dissociation, GDIs play a crucial role in the shuttling of Rho GTPases between the cytoplasm and membranes. For instance, Cdc42 function is also regulated by its subcellular localization, which depends on interactions with its GDI: active Cdc42 is bound to the membrane, whereas inactive Cdc42 is localized in the cytosolic fraction (17). Although much is known about the Rho-type GTPase structure and signal transduction, little is known about their regulation and function in immune cells. Rho GTPases have been demonstrated to regulate migration, and chemotaxis in monocytes and macrophages (18, 19). Endocytosis occurs via remodeling of the actin cytoskeleton and shares many of the core cytoskeletal components involved in adhesion and migration. Small GTPases of the Rho family have been implicated in coordinating actin dynamics in response to extracellular signals and during diverse cellular processes, including endocytosis, but the mechanisms controlling their recruitment and activation in DC are not known. Because it has been recently reported that DC developmentally regulate endocytosis at least in part by controlling levels of activated Cdc42 (3, 5), we have investigated in this study whether Rho GTPases are involved in the regulation of other DC functions. There are no published data on the role of Rho GTPases in the regulation of Ag presentation by DC. We report on the involvement of Cdc42, Rac, and Rag in Ag presentation by DC, using the OVA peptide as a model Ag. We have also investigated the role of the Rho-type small GTPases (Rac1, Cdc42, and RhoA) in regulating DC adhesion and motility, by transducing DC with constructs, encoding dominant-negative (dn) or constitutively active (ca) forms of Rho GTPases. We demonstrate that constitutive activation of Rho GTPases in DC differentially modifies DC Ag presentation, adhesion, chemotaxis, and endocytosis.

Materials and Methods

**Mice**

Male BALB/c (H-2Kd, I-Ad) mice, 6–8 wk old, were obtained from Taconic. Animals were maintained at Central Animal Facility at the University of Pittsburgh according to standard guidelines.

**DC generation**

Murine DC were generated as described previously (20). Briefly, mouse hemopoietic progenitors were isolated from bone marrow and depleted of RBC with lysing buffer (155 mM NaHCl in 10 mM Tris-HCl buffer (pH 7.5, 25°C). The single-cell suspension was incubated with a mixture of Abs followed by incubation with rabbit complement to deplete cells that express the lymphocyte Ags B220, CD4, and CD8. Cells were then cultured overnight (37°C, 5% CO2) in six-well plates (Falcon) at the concentration of 10^6 cells/ml in complete RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen Life Technologies). The nonadherent cells were collected and seeded at a concentration of 2 × 10^5 cells/ml in six-well plates in complete RPMI 1640 medium in the presence of recombinant mouse GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) (PeproTech). At day 6, DC were transduced with vaccinia virus (VV) encoding dn or ca forms of Rho GTPases (Cdc42, Rac, or Rho) proteins. Six hours later, DC were collected and used for further analyses.

**VV-based transduction of murine DC with the Rho family of small GTPases**

VV-based constructs, encoding dnCdc42, caCdc42, dnRac1, caRac1, dnRhoA, caRhoA, and control enhanced GFP (EGFP) and CD56 proteins were prepared as described earlier (21). The DC (5 × 10^6 cells) were transduced with VV-based constructs encoding dnCdc42, caCdc42, dnRac1, caRac1, dnRhoA, or caRhoA. VV was used at a multiplicity of infection of 5. For the transfection, DC were harvested on day 6, washed twice in HBSS, and incubated at 37°C for 4–6 h with corresponding VV-based construct. The VV/EGFP construct was used to check the efficiency of VV-based transfection by flow cytometry analysis. Transduction efficiency of VV/Rho GTPase-transduced DC was also determined by Western blot and immunocytochemistry.

In addition, we have used a Rho family GTPase inhibitor, toxin B (ToxB) (Calbiochem), which ribosylates ADP and inactivates Rho, Rac, and Cdc42 (22, and 2) a Rho family GTPase activator, cytoxic necrotizing factor 1 (CNF1) from Escherichia coli, which deamidates glutamine 63 of RhoA or 61 of Rac and Cdc42, rendering 2 ca GTPases (23). In some experiments, PMA was also used as a non-specific Rho GTPase activator (23). Before the functional assays, DC were pretreated with 1 ng/ml ToxB for 60 min, 250 ng/ml CNF1, or 60 ng/ml PMA for 30 min or medium (control).

**p21-activated kinase-1 (PAK-1) p21-binding domain pull-down assay**

Affinity purification assays that monitor Cdc42, Rac, and Rho protein activation are based on the fact that Rho proteins act as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state (24, 25). The Cdc42/Rac Activation Assay kit has been used in our experiments to assess specific binding and precipitation of GTP-Cdc42 and GTP-Rac from DC lysates (Upstate Biotechnology). The assay uses the Cdc42/Rac interactive binding (CRIB) region (also called the p21 binding domain (PBD)) of the Cdc42/Rac effector protein, PAK-1. The CRIB/PBD protein motif has been shown to bind specifically to the GTP-bound form of Rac or Cdc42, thus allowing isolation of active Cdc42 and Rac proteins. In addition, Rho Activation Assay kit has been used for precipitation of GTP-Rho from DC lysates (Upstate Biotechnology). This assay uses the Rhotekin Rho binding domain (Rhotekin RBD), which specifically binds to and precipitates GTP-Rho (active Rho), but not GDP-Rho (i.e., inactive Rho) from the cell lysates (26). The facts that the PBD region of PAK and RBD region of Rhotekin have a high affinity for GTP-Cdc42, GTP-Rac, and GTP-Rho, respectively, and that PAK and Rhotekin binding results in a significantly reduced intrinsic and catalytic rate of hydrolysis of Cdc42, Rac, and Rho make it an ideal tool for affinity purification of GTP-Cdc42, GTP-Rac, or GTP-Rho from cell lysates (27). Therefore, 5–10 μg of PAK-PBD agarose was added to 1 ml of cell lysate (200–1000 μg of protein) from DC transduced with VV-based (dn) or (ca) mutant forms of Rho GTPases for isolation of GTP-Cdc42, GTP-Rac, or GTP-Rho. Reaction mix was gently rocked at 4°C for 60 min. The Rac and Cdc42 proteins bound to PAK-1 PBD, and Rho protein bound to Rhotekin RBD were separated by SDS-PAGE. Proteins were transferred to polyvinylidene di-fluoride membrane and probed with specific anti-Rac, -Cdc42, and -Rho Abs (Upstate Biotechnology and BD Transduction Lab). The secondary Abs were HRP conjugated (1:100,000; Pierce). The immunoblot was processed and treated with chemiluminescent reagents (Pierce), and the bands were visualized on Kodak film (Eastman Kodak).

**Western blot and immunocytochemistry**

Expression of total Cdc42, Rac1, and RhoA proteins in DC lysates prepared after VV-based transfections, was determined by Western blot. The cell lysates from nontransfected and VV/CD56-transfected DC were used as controls. For the Western blot and immunocytochemical detection of tested small Rho GTPases, the following primary Abs were used: mouse anti-Cdc42 (1:250; BD Transduction Lab), mouse anti-Rac (2 μg/ml; Upstate Biotechnology), and mouse anti-RhoA (3 μg/ml; Upstate Biotechnology).

Percentage of DC overexpressing Cdc42, Rac1, or RhoA after VV-based transfection was determined by immunocytochemistry. Nontransfected DC and CD56-transfected DC served as controls. Immunocytochemical analysis of Cdc42, Rac1, and Rho expression was performed on the cytosplendid (100 × g; 5 min) cells, which were air-dried, fixed in ice-cold acetone, and permeabilized with saponin.

**Ag presentation assay**

Murine bone marrow-derived DC were generated in cultures with GM-CSF and IL-4 and transduced with VV encoding dn or ca mutant forms of Cdc42, Rac, and Rho, or control protein as described above. To evaluate DC ability to present a specific Ag, we tested capacity of modified DC to present OVA (OVA-derived antigenic peptides) to syngeneic specific T cells. Ag presentation was assessed by the IL-2 production by the specific OVA-recognizing T cell clone DO11.10 obtained from DO11.10 transgenic mice (H-2Kd, I-Ad) expressing the DO11.10 TCR that is specific for...
the peptide fragment of OVA323–339 in the context of I-A<sup>E</sup>. This clone was kindly provided by Dr. B. Osborne (University of Massachusetts, Amherst, MA). All groups of transduced DC (5 × 10<sup>5</sup> cells/ml) were pulsed with dialyzed whole OVA (Sigma-Aldrich; 1 mg/ml, experimentally established optimal dose for IL-2 production) for 24 h, collected, washed, and treated with medium (control), CNF1 (250 ng/ml; 30 min) or ToxB (1 ng/ml; 60 min). Then, DC were washed again and mixed (5 × 10<sup>5</sup> cells) with syngeneic T cell clone DO11.10 (2 × 10<sup>5</sup> cells) in triplicate in 96-well U-bottom plates. Cell-free supernatants were collected 48 h later, and concentrations of IL-2 were determined by ELISA (Endogen) according to the manufacturer’s instructions.

**Cell adhesion assay**

To evaluate the effect of Rho GTPases on DC adherence to extracellular matrix in vitro, DC transfected with VV encoding dn or ca mutants of Cdc42, Rac, and Rho were washed and counted. In addition, CNF1 and ToxB treatment was also performed as described above. A total of 2 × 10<sup>4</sup> cells in 120 μl of complete RPMI 1640 medium was placed in different wells on eight-chamber microscope slide coated with polylysine (Fisher Scientific). After 2-h incubation at 37°C, medium was gently aspirated, chambers were washed with warm medium, and disassembled, and adherent cells on the slides were fixed and stained with LeukoStat staining kit (Fisher Scientific). Enumeration of adherent DC was performed in at least 10 high power fields (HPF) using light microscopy independently by two investigators.

**Chemotaxis assay**

Cell migration was measured in 48-well Transwell plates (5-μm pores; Corning Costar). Recombinant murine MIP (MIP1α; 10 ng/ml; PeproTech) and synthetic chemotactic peptide fMLP (10<sup>−7</sup> M; Sigma-Aldrich) were diluted in RPMI 1640 medium containing 1% FBS (assay medium), and 600-μl aliquots were placed in the lower chamber of Transwell plates. Assay medium was used to measure spontaneous migration of DC. All groups of transfected DC were diluted at 1 × 10<sup>5</sup> cells/ml, and 100 μl of cell suspension was added to each Transwell insert. After 4-h incubation at 37°C, the Transwell inserts were removed, and cells from the lower chamber were collected. Cells transmigrated through the 5-μm pore size membrane were acquired on FACSscan (BD Biosciences) for 60 s. Data are reported as mean numbers of transmigrated cells from duplicate wells.

**Endocytosis assay**

The regulation of endocytotic activity of DC by the Rho family GTPases was assessed by FITC-dextran-40 (2 mg/ml; Molecular Probes) uptake in control DC and DC pretreated with 250 ng/ml CNF1 for 30 min, 60 ng/ml PMA for 30 min, 1 ng/ml ToxB for 60 min, or medium (control). Active endocytosis of dextran 40 was measured for 30 min at 37°C, whereas background diffusion was determined at 4°C. Positive cells were analyzed by flow cytometry.

**Statistical analysis**

For a single comparison of two groups, Student’s <i>t</i> test was used after evaluation for normality. If data distribution was not normal, a Mann-Whitney rank sum test was performed. For the comparison of multiple groups, one- or two-way ANOVA was applied. For all statistical analysis, the level of significance was set at a probability of 0.05 to be considered significant. All experiments were repeated at least two to three times. Data are represented as the mean ± SEM.

**Results**

**Expression of Rho GTPases in murine DC**

The most common method to investigate Rho GTPases in cells is to microinject dn or ca forms into tested cells (9, 18, 28). However, this methodology cannot be used to study certain functions of DC due to the great number of cells, such as Ag presentation, adhesion, etc. To solve this problem, we have established a new method of DC modification using a vector-based approach with VV-based constructs encoding dnCdc42, caCdc42, dnRac, caRac, dnRho, caRho, and control EGFP and CD56 proteins. First, using VV/EGFP, we have demonstrated that transduction efficacy of DC reaches 82% as was determined by a FACSscan analysis (Fig. 1). Protein expression was maximal in 4–8 h.

Next, expression of total Cdc42, Rac1, and RhoA proteins in DC lysates prepared after VV-based transfections, was also determined by Western blot and immunocytchemistry. The results revealed that, as expected, DC from all experimental groups express detectable levels of endogenous total Cdc42, Rac1, and RhoA. The variably expressed proteins were distinguished from endogenous constitutively expressed Cdc42, Rac1, and RhoA proteins by the appearance of the slower migrating specific bands containing myc-tag epitopes (21). The results of these studies are shown in Fig. 2 and demonstrate 1) high levels of expression of virally encoded (ca) and (dn) mutant forms of Cdc42, Rac1, and RhoA and 2) comparable levels of expression of both mutant forms of Cdc42, Rac1, and RhoA. It is important to note that there are slight differences in the intensity of positive Western blot bands between Cdc42, Rac1, and RhoA, which are due to the different affinity/avidity of specific Abs used in the described assays. Finally, percentage of DC overexpressing Cdc42, Rac1, or RhoA after VV-based transfection was determined by immunocytchemistry. We demonstrated that ~70–80% of DC were strongly positive for Cdc42, Rho, or Rac staining after their transfection with VV encoding dnCdc42, caCdc42, caRac1, caCdc42, dnRac1, or caRho, respectively. In contrast, most of control DC exhibited only the background level of positive staining with no more than 20–30% of DC displaying low-to-medium level of positivity.

Next, we demonstrated that VV/caCdc42-, VV/caRac-, and VV/caRho-transduced DC express high levels of active GTP-bound forms of Rho GTPases Cdc42, Rac1, and RhoA (Fig. 3). In contrast, all three dn mutant forms of Cdc42, Rac1, RhoA, as well as control transfected and nontransfected DC did not exhibit any detectable binding to the fusion proteins, suggesting the absence or very low levels of active GTP-bound forms of Cdc42, Rac1, and RhoA. It is important to note that there are slight differences in the intensity of positive Western blot bands between Cdc42, Rac1, and RhoA, which are due to the different affinity/avidity of specific Abs used in the assays. However, the results of these studies demonstrate that 1) DC were efficiently transduced with ca and dn forms of all tested small Rho GTPases and 2) transfection was functional, i.e., (ca) mutants express active Rho GTPases, whereas (dn) mutants and control nontransfected DC express no active GTP-bound proteins. Thus, VV transfection of DC with Rho GTPases can be used to study the regulation of DC function.

In the next series of experiments, we evaluated whether transduction of DC with dn or ca mutant forms of Rho GTPases regulates DC adherence, migration, chemotaxis, endocytosis, and Ag presentation.
Regulation of DC adhesion by small GTPases from the Rho family

The results demonstrating the effect of Rho GTPases on DC adhesion in vitro are shown in Fig. 4. Thus, the Rho GTPase activator CNF1 significantly up-regulates adherence of both control (non-transduced) and CD56 (control protein)-transduced DC (23.6 ± 3.4 and 18.5 ± 2.3 vs 13.2 ± 1.7 and 14.1 ± 0.8 cells/HPF, respectively; p < 0.05), whereas Rho GTPase inhibitor ToxB significantly inhibits DC adhesion in both groups (7.0 ± 0.5 and 5.2 ± 0.4 cells/HPF; p < 0.05). ToxB also inhibited adhesion of the caCdc42-transduced DC (6.9 ± 1.1 cells/HPF; p < 0.05) (Fig. 4A). Transduction of DC with dnCdc42 markedly decreased (5.5 ± 0.4 cells/HPF), whereas transduction with caCdc42 strongly increased (19.5 ± 1.8 cells/HPF) adhesion of DC (p < 0.05). Furthermore, CNF1 only slightly up-regulated DC adhesion, which was blocked by the overexpression of dnCdc42, whereas ToxB did not further decrease it. Taken together with the fact that CNF1 did not further up-regulate adhesion of caCdc42-transduced DC, these data suggest that Cdc42 plays a major role among Rho GTPases in the regulation of DC adhesion. This was confirmed in the second set of experiments where the effect of the different Rho GTPases on DC adhesion was compared (Fig. 4B). The transduction of DC with caRho caused only a modest elevation of DC adherence (14.4 ± 1.1 vs 8.8 ± 0.8 in control; p < 0.05). This effect was blocked by ToxB, but not by caRac. Thus, among the three tested Rho GTPases, activation of Cdc42 in DC displayed the highest potential for augmenting DC function.

Regulation of Ag presentation in DC by the Rho family GTPases

In the next series of studies, we have used the same approach to activate or block Rho GTPase in DC (Fig. 5A). The results show that CNF1 increased, whereas ToxB decreased the ability of DC to present OVA-derived peptides to OVA-specific T cell clones, as determined by IL-2 production by activated T cells (359 ± 40 and 128 ± 10 vs 248 ± 26 pg/ml/48 h in controls; p < 0.05). Both caCdc42 and caRho up-regulated Ag presentation by DC (up to ~450 pg/ml), which could be blocked by ToxB. dnCdc42 and dnRho decreased Ag presentation, although the effect of dnRho did not reach statistical significance. Surprisingly, both ca and dn forms of Rac blocked DC Ag presentation (p < 0.05), suggesting that Rac might mediate different effector pathways in DC than Cdc42 and Rho. Together, these data demonstrate that Rho GTPases are involved in Ag presentation by DC, although the effector mechanisms remain to be elucidated.

Regulation of DC motility by the Rho family GTPases

The effects of Rac, Rho, and Cdc42 on DC chemotaxis and spontaneous migration in a Transwell system are shown in Fig. 5B. Constitutive activation of Cdc42 in DC significantly inhibits both spontaneous (7,380 ± 568 vs 12,260 ± 1,112 cells/min in control; p < 0.05) and chemokine-induced DC migration: 8,040 ± 590 vs 30,240 ± 2,674 cells/min for chemotactic peptide fMLP and 9,780 ± 867 vs 39,414 ± 4,532 cells/min for CC chemokine MIP1α (p < 0.001). Interestingly, the dnCdc42 mutant did not modulate DC migration in either assay. Unexpectedly, all tested mutant forms of Rac and Rho (except dnRAC) significantly inhibit both spontaneous migration and chemotaxis of transduced DC.

Together, our results demonstrate differential requirements for the Rho family GTPases in DC motility and suggest that a dynamic regulation of Rac and Rho may be required for DC chemotaxis.

Regulation of endocytotic activity of DC by the Rho family GTPases

Because it has already been reported that DC developmentally regulate endocytosis by controlling levels of activated Cdc42 (5), we next demonstrate that Rho GTPases are involved in the regulation of DC endocytotic activity in our test system (Fig. 6). The results indicate that uptake of dextran 40, which reflects mannose receptor-mediated endocytosis in DC, was significantly up-regulated (up to 170% and up to 144%) by the Rho GTPase activators CNF1 and PMA, respectively (Fig. 6), and reduced 2- to 4-fold by a Rho GTPase inhibitor, ToxB (Fig. 6, p < 0.05). Our results also reveal that transduction of DC with caCdc42 increases their endocytotic...
Regulation of DC adhesion by small GTPases from the Rho family. Murine bone marrow-derived DC were generated in cultures with GM-CSF and IL-4 and harvested on day 6. After washing, DC were transduced with VV encoding dn or ca mutants of Cdc42, Rac and Rho. Four hours later, DC were washed, and 2 × 10^5 cells in 120 µl of complete medium were placed on a microscope slide with 8 chambers. After 2-h incubation at 37°C, medium was aspirated, and adherent cells were fixed and stained with LeukoStat staining kit. Enumeration of adherent DC was performed in at least 10 HPF using light microscopy. The results are shown as the mean ± SEM (n = 3). A, Modulation of Cdc42-mediated effect on DC adhesion by Rho GTPase inhibitor ToxB (1 ng/ml) and Rho GTPase activator CNF1 (250 ng/ml). * p < 0.05 (one-way ANOVA). B, Inhibition of Rho GTPase-induced up-regulation of DC adhesion by ToxB. * p < 0.05 (one-way ANOVA).

In summary, our data demonstrate that DC adhesion, Ag presentation, migration, chemotaxis, and endocytosis are differentially regulated by the Rho family GTPases.

Discussion
Rho-related small GTPases, including Cdc42, Rac, and Rho, are known to regulate actin reorganization in response to different extracellular cues in several adherent cell types (29, 30). Recently, other cellular functions have also been ascribed to these proteins, i.e., transcriptional regulation, growth control, endocytosis, and exocytosis (5–7). However, little is known about the role of small Rho GTPases in the regulation of DC function. Thus, we have investigated the role of Rho GTPases in regulating DC adhesion, Ag presentation, migration, chemotaxis, and endocytosis. Rho family proteins are known to regulate cell adhesion in macrophages and fibroblasts. The most common method to examine the role of Rho GTPases in cells is to microinject dn or ca forms into test cells (18). For example, Allen et al. (31) demonstrated that using Bac1 macrophages, Cdc42 and Rac are required for the assembly of adhesion sites to the extracellular matrix. Cdc42 has been implicated in VLA-4-dependent adhesion of macrophages (19). Rac2 appears to be critical for hemopoietic stem cell adhesion both in vitro and in vivo (32). In this study, we demonstrate for the first time that the Rho GTPase activator CNF1 significantly up-regulates adherence of both control (nontransduced) and CD56 (control protein)-transduced DC, whereas a Rho GTPase inhibitor ToxB significantly inhibits DC adhesiveness in both groups. Furthermore, we showed that Cdc42 played a major role among other Rho GTPases in the regulation of DC adhesion in vitro, because transduction of DC with caCdc42 strongly increased DC adhesion to extracellular matrix. Rho GTPase inhibitor ToxB significantly down-regulates adhesion of caCdc42-transfected DC, demonstrating a specificity for this effect. However, transduction of DC with caRho caused only a modest elevation of DC adhesion, and caRac had no effect.

This effect may be explained by the formation or contraction of dendrites by different Rho GTPases. For example, Swetman et al. (9) showed that dendrite formation is directed by members of the Rho GTPase family, with the Cdc42 acting predominantly to promote cellular extension and spreading, and Rho, conversely, acting to drive contraction and detachment of the dendrites.
a specific Rho inhibitor exoenzyme C3 from Clostridium botulinum and a specific Rho-associated kinase inhibitor Y-27632, it has been demonstrated that both C3 and Y-27632 markedly reduce actin polymerization in parallel with the disappearance of dendrites in DC (7, 33). Furthermore, DC microinjected with a ca mutant of Cdc42, which lacks intrinsic GTPase activity, show exaggerated filopodial activity, whereas dnCdc42, which depletes the cell of guanine nucleotide exchange factors, inhibits spike formation and membrane ruffles (28).

Next, we have demonstrated the role of Rho GTPases in the regulation of MHC class II Ag presentation and chemotaxis in DC. To evaluate the ability of DC to present specific Ag, we have tested the capacity of dnCdc42-, caCdc42-, dnRac-, caRac-, or cnRho-transduced DC to present OVA peptides to the specific OVA-recognizing T cell clone DO11.10 obtained from transgenic mice expressing the TCR specific for the OVA\textsubscript{323–339} peptide. We have shown that CNF1, an activator of small Rho GTPases, increased, whereas ToxB, an inhibitor of Rho GTPases, decreased the ability of DC to present the OVA to specific T cells. Furthermore, caCdc42 and caRho increased Ag presentation by DC, and the effect was prevented by ToxB. dnCdc42 and dnRho decreased Ag presentation. Surprisingly, both ca and dn forms of Rac blocked DC Ag presentation, which suggests that Rac may serve as a down-regulator of Cdc42 and Rho either directly or indirectly. It is possible that increased Ag presentation by DC with activated Rho GTPases is mediated by increased endocytosis. It is known that DC developmentally regulate endocytosis by controlling levels of activated Cdc42 (5). We have confirmed these data by showing that uptake of FITC-dextran, i.e., receptor-mediated endocytosis, was significantly up-regulated by Rho GTPase activators CNF1 and PMA and reduced by Rho GTPase inhibitor ToxB. Transfection of DC with caCdc42 increased their endocytotic activity, whereas transfection with dnCdc42 decreased it. Similarly, it was reported that treatment of immature spleen-derived DC (8) or bone marrow-derived DC (5) with ToxB completely blocked the macropinocytotic uptake of fluorescent fluid-phase markers, a form of high-volume, nonspecific endocytosis involving the extension of membrane ruffles. The involvement of specific Rho family members was dissected by microinjection of dn inhibitory versions of the different Rho GTPases. Administration of dnRac1 or dnCdc42 blocked macropinocytosis in the immature DC. Significantly, in mature DC, which have down-regulated macropinocytosis, microinjection of activated Cdc42 stimulated endocytosis, indicating that the machinery for macropinocytosis controlled by Cdc42 remains intact after maturation (5). These results implicate both Cdc42 and Rac in the regulation of endocytosis in DC and strongly suggest that the shutdown in endocytosis seen during maturation is caused by inactivation of Cdc42 (6). Thus, Cdc42-mediated up-regulation of endocytosis may play a role in increased Ag presentation by DC. Additional mechanisms are also likely to be involved. For instance, using a specific Rho inhibitor exoenzyme C3 and a specific Rho-associated kinase inhibitor Y-27632, it has been demonstrated that inactivation of Rho in DC was associated with inhibited interaction between DC and CD4\textsuperscript{+} T cells and ~80% reduction of T cell stimulatory capacity in allogeneic MLR, although the surface expression of MHC, co-stimulatory, and adhesion molecules were unaffected (7, 33). Finally, because intracellular transport of secretory vesicles and exocytosis is also regulated by small Rho GTPases (34–36), one can hypothesize that transport of MHC class I/peptide complexes to the cell surface and increased production of DC cytokines may up-regulate T cell activation by DC as well. However, this hypothesis requires further experimental verification.

Activation of Cdc42 in DC significantly inhibited spontaneous and chemokine-induced (fMLP and MIP1\textalpha) DC migration. Interestingly, the dnCdc42 mutant did not modulate DC migration, which suggests that the inhibitory effect of active Cdc42 may involve activation of other members of the Rho family known to play a role in the regulation of chemotaxis and migration in other cell types. Unexpectedly, all tested mutant forms of Rac and Rho (except dnRac) significantly inhibited both migration and chemotaxis of transduced DC. Taken together, these data suggest a complex mechanism and multiple roles for Rho GTPases in the regulation of DC chemotaxis and migration. This is in accordance with the published data on macrophages, where Allen et al. (18) have recently reported that Rho and Rac are required for the process of macrophage migration, whereas Cdc42 is required for cells to respond to chemokines but is not essential for cell locomotion.
and migration. The authors also reported that caRho, caRac, and caCdc42 reduce macrophage translocation, and/or migration and activated Rho may act antagonistically to Rac and Cdc42. Similarly, inhibition of endogenous Rho and Rac proteins also prevents chemokine-induced cell migration, both in DC and in macrophages (18). In another report, Weber et al. (19) have demonstrated that CC chemokines, including MIP1α, or caCdc42 induce formation of filopodia-like projections in monocytes. Both caCdc42 and dnCdc24 mutants inhibited CC chemokine-induced monocyte migration, implicating Cdc42 activity and its effector functions in chemotaxis (19). Cdc42 has been also involved in membrane ruffling induced by IMLP and PMA (37).

Additional evidence of the important role of Rho GTPases in DC function was obtained from the investigation of DC harvested from Wiskott-Aldrich syndrome protein (WASP)-null animals and WASP-null humans. WASP is a member of a recently defined family of proteins that are involved in the transduction of signals to the actin cytoskeleton (28). WASP is uniquely expressed on hemopoietic cells, and is the specific effector of Cdc42 (38). WASP-deficient DC have cytoskeleton (28). WASP is uniquely expressed on hemopoietic cells, and is the specific effector of Cdc42 (38). WASP-deficient DC have...