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Lipid Raft-Associated GTPase Signaling Controls Morphology and CD8⁺ T Cell Stimulatory Capacity of Human Dendritic Cells

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Their eponymous morphology and unique ability to activate naive T cells are hallmark features of dendritic cells (DCs). Specific properties of the actin cytoskeleton may define both characteristics. In search for regulators that coordinate DC phenotype and function, we observed strongly increased expression of the actin-remodeling GTPases Cdc42 and Rac1 during DC development from human stem cells. Cdc42 and Rac1 are constitutively active in immature DCs, and their activity is further up-regulated by maturational stimuli such as LPS or CD40L. Activation of Rac1 is associated with its rapid recruitment into lipid rafts. Cdc42 is not recruited into rafts, but readily activated by raft-associated moieties. The functional interplay of rafts, GTPases, and cortical actin is further shown by GTPase activation and actin remodeling after pharmacological disruption of lipid rafts and by the loss of the actin-based DC morphology by transfection of dominant-negative Cdc42 and Rac1. Both Cdc42 and Rac1 also control the transport of essential immunostimulatory molecules to the DC surface. Transfection with dominant-negative GTPases led to reduced surface expression of MHC class I and CD86. Consequently, DCs display a reduced stimulatory capacity for CD8⁺ T cells, whereas MHC class II-dependent stimulation of CD4⁺ T cells remains unperturbed. We conclude that Cdc42 and Rac1 signaling controls DC morphology and conditions DCs for efficient CD8⁺ T cell stimulation. The Journal of Immunology, 2004, 173: 1628–1639.

Dendritic cells (DCs) differ from all other cell types by their unique ability to elicit primary T cell responses. In their immature state, DCs reside at sites of Ag exposure such as the mucosal surfaces and the skin. These DCs have a high capacity to endocytose and process exogenous Ags. Upon exposure to innate stimuli (e.g., TLR ligands), DCs undergo a process of functional maturation that includes the termination of Ag uptake, the migration from the periphery to secondary lymphoid organs, the up-regulated surface expression of MHC Ags and co-stimulatory molecules, and the secretion of cytokines such as IL-12. Mature DCs are capable of productively presenting previously captured antigenic material to naive T cells (1). Cognate contact with CD4⁺ T cells in lymphoid organs leads to CD40L-dependent activation of DCs. CD40L-stimulated DCs continue to up-regulate MHC products and CD86 and become empowered to induce efficient CD8⁺ T cell responses by yet not completely resolved molecular mechanisms (2, 3).

DC maturation is also associated with an increase in cellular dendricity. Typically, the surface of immature and, to a much greater extent, mature DCs is constitutively ruffled, bearing numerous filopodia and lamellipodia/veils (4), while in other cell types, such morphological features are only temporarily inducible (5). These cytological features are best visible in DCs isolated from organs and cultured under maturation-promoting conditions in vitro or in DCs grown from stem cells in cytokine-conditioned medium. Besides the filamentous processes mentioned above, DCs project long arborizing dendrites during their residence in peripheral organs (e.g., in the epidermal compartment of the skin). It is tempting to speculate that the functional role of the filamentous processes of DCs relates to optimized Ag uptake and cell-cell communication. Thus, one may suspect that the peculiar surface architecture of DCs promotes the likelihood of productive DC-T cell encounters and is thus linked to the extraordinary stimulatory capacity of these cells. The mechanisms regulating these autonomous and maturation-related DC-specific actin rearrangements remain to be elucidated in detail.

Other key features of APCs, such as their phagocytic or macrophagic Ag uptake, cell migration, and intracellular vesicle transport/membrane trafficking, are as well actin-dependent processes (6). Consequently, the identification of the factors regulating the various aspects of actin modeling in DCs will improve the understanding of the basic principles of different functional capabilities of this cell type.

Rho family GTPases are known to orchestrate actin reorganization (7), and are thus candidates for the control of actin-dependent...
DC functions. Small GTPases cycle between an inactive GDP-bound and an active GTP-bound state. Actin polymerization induced by the small GTPases Cdc42 and Rac1 is executed by members of the Wiskott-Aldrich syndrome protein/Vesprin-Homologous Protein family, which stimulate the Arp2/3 complex to nucleate new actin filaments (6, 8). Existing data on GTPase expression and function in DCs to date suggest that Cdc42 and Rac1 activity is critical for polarization and motility of monocyte-derived DCs (9, 10). Moreover, Cdc42 and Rac1 were found to be both required for the formation of long filamentous processes that DCs form upon adhesion to extracellular matrix-coated surfaces (10). In the same adhesion-based system, inhibition of p160ROCK, a downstream effector kinase of RhoA, led to the formation of longer dendrites due to a defect in cellular contraction and adhesion regulation (10). Conversely, treatment of monocyte-derived DCs with the Rho GTPase inhibitor exoenzyme C3 was found to induce the loss of the constitutive filamentous surface projections of DCs and to reduce the alloantigen-presenting capacity of these cells (11). However, exoenzyme C3 not only inhibits the GTPase Rho, but presumably also induces actin depolymerization of F-actin by removing the cofillin-dependent brake for F-actin decay (12). Thus, data obtained by use of exoenzyme C3 speak in favor of an important role of the DC’s F-actin cytoskeleton without addressing the regulatory role(s) of individual GTPases. LPS exposure of immature bone marrow-derived murine DCs down-regulates active Cdc42 (13), while the same stimulus increases Cdc42 activity in murine splenic DCs (14). Rac1 (14) or Cdc42 (13) was found to be essential for the constitutive macrocytosis of immature DCs. In the latter study, a down-regulated Cdc42 activity was functionally linked to the loss of macrocytotic activity in mature DCs (13). It is not decided whether the apparent discrepancy may relate to fundamental differences in Cdc42 regulation in different DC subtypes or may be due to variations in the detection levels of activated GTPases when they are bound to downstream targets in activated/mature DCs (15).

An important role of Cdc42 and Rac1 in exocytotic vesicle transport is well established (6). Cdc42, as an integral member of the brefeldin A (BFA)-sensitive component of the Golgi apparatus, regulates protein export from the Golgi/ER-Golgi network (16–18). Given the activity of certain relevant GTPases during the late stages of DC maturation, a role of GTPases in the transport of MHC molecules and the regulation of MHC-dependent Ag presentation appears possible. Besides regulating protein trafficking, GTPase-dependent actin polymerization may also regulate the export of lipid domains to the cell surface. In mast cells, evidence exists that Cdc42 plays an important role in the biosynthesis of lipid raft components (19). In fibroblasts, actin polymerization contributes to the transport of Golgi-derived lipid rafts to the plasma membrane, where these microdomains link the actin cytoskeleton to the cell membrane (20). Lipid rafts are critically involved in the signal transduction during the initiation of immune responses (21). In T cells, Vav-, Rac-, and Wiskoff-Aldrich syndrome protein-dependent act reorganization induces lipid raft clustering, which is considered critical for signaling after the interaction with APCs (22, 23). In DCs, lipid rafts are still poorly investigated. Recently, DC rafts have been shown to contain a subset of MHC class II-peptide complexes (24). Signals transmitted via DC rafts were shown to condition the DC for the activation of CD8+ T cells (25). It is tempting to speculate that small GTPases likewise regulate the export of lipid rafts and of their components to the DC surface and thereby control phenotype and function of DCs.

In summary, a critical role of the act cytoskeleton for some functions of mature DCs has been described, but the role of specific GTPases is still poorly understood. In this study, we tried to define the functional roles of individual GTPases for the morphology and the Ag-presenting capacity of DCs grown from human stem cells in vitro. Microinjection of DCs with GTPase mutants, the commonly used experimental model for GTPase regulation (13, 14, 26), was no option for long-term studies with primary DCs and T cells, because only small cell numbers can be modified, and the effect of injected GTPases is short-lived only. In this study, we used a transfection system for CD34+ stem cell-derived Langerhans cell (LC)-type DCs (referred to as DCs from now on) that allows sustained expression of transgenes. Transfections were performed with a bicistronic expression vector that defines modified DCs by enhanced GFP (EGFP) expression. This system was used to elucidate whether and how Cdc42 and Rac1 regulate morphology and function of human DCs.

**Materials and Methods**

**Abs and reagents**

FITC-conjugated mAbs included: anti-CD1a (OKT6; Ortho Diagnostics, Raritan, NJ), and anti-CD14 (macrophage P9; BD Neon) from both DC Biotechnology, Gaithersburg, MD, and R&D Systems, Minneapolis, MN. Alexa Fluor647- or Alexa Fluor633-labeled goat F(ab’2) anti-mouse IgG and streptavidin (SA) Alexa Fluor647 or Alexa Fluor633 from Molecular Probes (Eugene, OR), SA-PE was from BD Biosciences. Tetramethylrhodamine isothiocyanate (TRITC)-conjugated cholera toxin subunit B (Ctx B) was from List Biological Laboratories (Campbell, CA). TRITC-coupled phallolidin was from Sigma-Aldrich. LPS (Salmonella typhimurium), BFA, n-octylglucoside (NOG), methyl-β-cyclodextrin (MβCD), and cholesterol were from Sigma-Aldrich. Latrunculin (Lat B) was from Calbiochem (Darmstadt, Germany).

**Generation of LC-type DCs and Mfb from CD34+ stem cells**

CD34+ hematopoietic stem cells (HSCs) were separated from cord blood mononuclear cells by positive immunoselection (Direct CD34+ Progenitor Cell Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in RPMI 1640/10% FCS supplemented with GM-CSF (200 U/ml)/TNF-α (50 U/ml)/Flt3-L (50 ng/ml), as described (27). On day 6, cultures were split 1:3, and fresh GM-CSF/TNF-α-containing medium supplemented with TGF-β1 (final concentration: 1 ng/ml) was applied to promote the development of LC-type DCs (27). On day 10, cells were harvested and were either used for LPS (1 μg/ml) stimulation experiments or again placed in fresh GM-CSF/TNF-α-containing medium. Immunophenotyping at day 12 regularly revealed >80% CD1a+ CD14- e-cadherin+ MHC class II+ CD86 high LC-type DCs (data not shown). CD1a positivity was used as the benchmark criterion for DCs in all FACS-based immunophenotyping and cell-sorting experiments. To induce terminal DC maturation, cultures were substituted at day 12 with FLA-tagged CD40L (10 ng/ml) and anti-FLAG mAb (2 μg/ml) for >2 days. In certain experiments, DCs were harvested at the culture day indicated and recultured in serum-free medium supplemented with 5 μM Lat B (1 h), 10 mM MβCD (15 or 45 min), or 10 mM MβCD and cholesterol solution at 37°C. Viability of the cells was assessed by trypan blue staining. To generate Mfb, GM-CSF/TNF-α/Flt3-L-stimulated CD34+ HSC-derived cells were collected on day 6, reseded in fresh medium containing M-CSF (2500 U/ml) and IL-6 (200 U/ml), and cultured until day 12 (27). Immunophenotyping at day 12 regularly revealed that >90% of the cells were CD14+ CD1a- MHC class...
Transfection
DNAs encoding mve-tagged dominant-negative (N17 mutants) and constitutively active (L61 mutants) GTPases (Cdc42 and Rac1) were kindly provided by A. Hall (University College, London, U.K.) (28). Constructs were subcloned into pCMV-EGFP (BD Clontech, Palo Alto, CA). A total of 1 × 10^7 day 12 DCs or Mφ were electroporated with 10 μg of DNA in 800 μl of RPMI 1640 at 960 μA/240 V. Thereafter, 3 × 10^5 cells/ml were replated in pretransfection medium, and transfection efficiency was determined by EGFP expression on day 13. CD1a^+EGFP^ cells were 12 ± 7% of vector control (n = 20), 14 ± 6% of N17Cd42 (n = 20), and 10 ± 6% of N17Rac1 (n = 20)-transfected DCs. Transfected Mφ (CD14^+EGFP^ cells) were obtained with an efficacy of 5 ± 4% for the vector control (n = 6), 5 ± 3% for L61Cd42 (n = 6), 35 ± 4% for L61Rac1 (n = 6).

For functional assays, transfected cells were harvested on day 13 and stained with PE-conjugated anti-CD1a or anti-CD14, as described (27). EGFP^-CD1a^- DCs or EGFP^-CD14^- Mφ were FACs sorted (FACStar™, BD Biosciences). Sorted cells were propagated in pretransfection medium either supplemented or not with CD40L until day 15. Recovery of EGFP^/propidium iodide^- cells was 45 ± 9% for vector control (n = 10), 43 ± 6% for N17Cd42 (n = 10), and 47 ± 5% for N17Rac1 (n = 10)-transfected DCs, and 30 ± 7% for control (n = 6), 35 ± 4% for L61Cd42 (n = 6), and 37 ± 5% for L61Rac1 (n = 6)-transfected Mφ after 2 days of culture.

Immunofluorescence microscopy
Day 10 DCs were allowed to adhere to adhesion slides (Bio-Rad, Richmond, CA), as described (29). Slide-bound DCs were exposed to 1 μg/ml LPS for the indicated time periods in RPMI 1640/10% FCS. Thereafter, cells were washed, fixed with 4% paraformaldehyde (Fluka, Buchs, Switzerland), and permeabilized in 0.1% Triton X-100 (Bio-Rad)/PBS before incubation with primary Abs was performed (2–20 μg/ml, 60 min at 4°C). Binding of mAbs was visualized with fluorochrome-conjugated secondary reagents (1–5 μg/ml). F-actin and the glycosphingolipid GM1 were detected by fluorochrome-labeled phalloidin (2 μg/ml). Cells were washed and cultured in 10 μg/ml respective reagents. Control stains were included in all experiments. After mounting (Prolong Antifade Kit; Molecular Probes), confocal laser-scanning microscopy (CLSM; LSM 510; Zeiss, Oberkochen, Germany) analysis was performed. Data analyses were performed using the manufacturer’s standard software package.

Flow cytometry analyses
For two- or three-color immunolabeling, 1–5 × 10^5 cells were incubated with fluorochrome-conjugated mAbs or isotype control Abs (2 μg/ml each) for 30 min on ice. Biotinylated reagents were detected with SA-PE. Fluorescence was analyzed on a FACScan (BD Biosciences).

For studies on the transport kinetics of conformationally intact MHC class I to the cell surface, DCs were transfected at day 12 and cultured in the presence of CD40L until day 14. DCs were then harvested, washed, and incubated with chilled elution buffer (131 mM citric acid, 66 mM Na_2 HPO_4, pH 3.3) for 1.5 min to remove surface MHC class I-bound β2-microglobulin (β2m) and peptides. Cells were washed and cultured in RPMI 1640/5% BSA at 37°C for the indicated chase periods. Harvested cells were washed, and 5 × 10^5 DCs were stained with mAb W6/32-PE, selectively recognizing conformationally intact MHC class Iβ2m/peptide complexes, and anti-CD1a Cy5, and analyzed by FACs. Transfected DCs were isolated as EGFP^/CD1a^- cells, and their CD40L immunoreactivity was recorded. Removal of pre-existing MHC class Iβ2m/peptide complexes was efficient (>85% reduction of W6/32 immunoreactivity by incubation in acidic elution buffer). Export of MHC class I to the cell surface is presented as the mean fluorescence intensity (MFI) of W6/32 immunoreactivity of DCs that were kept on ice.

MLR experiments
CD4+ or CD8+ T cells were purified from human cord blood as described (30). Graded numbers of sorted CD4+ DCs and CD14+ Mφ transfected with the indicated GTPase constructs or empty vector only were cultured together with 5 × 10^4 naive allogeneic CD4+ or CD8+ T cells in a 6-day MLR. In another set of experiments, DCs at day 10 were activated for 3 days with LPS or CD40L or were left nonactivated and then were used as stimulators for allogeneic CD4+ or CD8+ T cells. In experiments addressing co-stimulatory activity, 1 × 10^7 CD40L-stimulated transfected DCs were cocultured in a 4-day MLR with 5 × 10^5 naive autologous CD8+ T cells in the presence of various concentrations of plate-bound anti-CD3 mAbs.

IL-12 secretion
Flow-sorted EGFP^-CD1a^- DCs (purity >98%) were stimulated with CD40L for 2 days. Supernatants were collected and analyzed for IL-12p40 by ELISA, as described (31).

SDS-PAGE and Western blotting
Immunoblots were performed, as described (32). Blotted samples were probed with first-step reagents (anti-Cdc42, anti-Rac1, anti-MHC class I H chain, or anti-CD59), followed by HRP-coupled goat anti-mouse IgG (Bio-Rad) and West Pico luminescent reagent (Pierce, Rockford, IL). Where indicated, protein concentrations were measured (DC protein assay; Bio-Rad). The amount of active Cdc42 or Rac1 was defined as follows: band intensities were determined by MultiImage light cabinet ChemiImager 4400 (Alpha Innotech, Oldendorf Hess, Germany). Results obtained from total lysates (TL) were considered 100% for calculating the values of the corresponding lysates depleted of p21-activated kinase (PAK) 1-binding moieties (see below).

Sucrose density gradients
Day 10 DCs in single cell suspension were exposed to 1 μg/ml LPS for the indicated time periods in RPMI 1640/10% FCS. Cells were washed, and membrane rafts were isolated by sucrose density gradient ultracentrifugation, as described (33), with some modifications. Briefly, cells (2 × 10^7 cells/ml) were lysed with Mg^2+ /lysis/wash buffer (MLB; Upstate Biotechnology) supplemented with 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na-orthovanadate, and 25 mM NaF (all from Sigma-Aldrich) for 30 min at 4°C. Nuclear material was removed by brief centrifugation (13,000 rpm, 20 s, 4°C). Supernatant (275 μl) was mixed with an equal volume of 80% sucrose solution (in MLB) and placed on bottom of a 5–20% linear sucrose gradient, and samples were centrifuged at 345,000 × g (16 h, 4°C). Fractions of 400 μl were taken from top of the gradient, mixed with SDS-PAGE sample buffer, and subjected to SDS-PAGE and Western blotting. Virtually identical results were observed in fractionation and Western blotting experiments with cells lysed in MLB lysis buffer, 1% Brij-58, or 0.1% Triton X-100 (data not shown).

PAK 1-based pull-down (PD) assays for GTPase activity
GTP-bound Cdc42 or Rac1 was precipitated with PAK1/PAK 1-binding domain (PBD)-coupled agarose (Upstate Biotechnology), following the manufacturer’s protocol. In brief, DCs were chilled, harvested, and washed twice with cold PBS. Cells (1 × 10^7 cells/ml) were lysed with MLB supplemented with protease inhibitors for 30 min at 4°C. Lysates were centrifuged to deplete cell debris (1500 rpm, 5 min, 4°C). Where indicated, postnuclear supernatants were incubated with 60 mM NOG for 20 min to destroy lipid rafts. In a different set of experiments, raft fractions (5–9) and soluble fractions (10, 11) were pooled or kept separate. Fractions were briefly incubated at room temperature and subjected to PAK 1 precipitation described below. Supernatant (500 μl) was incubated with 20 μg of PAK 1/PBD-coupled agarose for 60 min at 4°C to immobilize active Cdc42 and Rac1. As a positive control, lysates were treated with 1 mM EDTA in the presence of 1 mM GTPγS (15 min, 30°C). The GTP-binding reaction was stopped by addition of MgCl2 (final concentration 60 mM) before precipitation, and the PAK 1-depleted lysate was recovered. Pellets were washed three times, and precipitated proteins were eluted with nonreducing SDS-PAGE sample buffer. Adjusted aliquots of TL, PAK 1-depleted lysates, and precipitated proteins were separated by 15% SDS-PAGE and analyzed by Western blotting.

Results
Cdc42 and Rac1 GTPases are constitutively active in immature DCs and superactivated by maturation-inducing stimuli
In vitro differentiation of DCs from CD34+ cord blood stem cells provides a valid model of DC ontogeny in vivo (27). DCs begin to develop filopodia and lamellipodia after day 6 of culture. DCs exposed after day 10–12 to maturation stimuli such as CD40L develop uniformly the dendritic shape typical of mature DCs (4) (data not shown). We analyzed the protein levels of actin-remodeling GTPases during DC ontogeny by Western blotting of normalized amounts of total cellular protein (Fig. 1A). Low levels of Cdc42 and Rac1 are detectable on day 6. Thereafter, Cdc42 and...
Rac1 are up-regulated, and robust expression is detected from day 9 to 14 (Fig. 1, A and B). Although Rac1 expression remains essentially unchanged after day 9, Cdc42 protein levels continue to increase until day 14 (Fig. 1B). PAK 1-based pull-down assays define a pool of constitutively active, GTP-bound Cdc42 and Rac1 from days 10 to 14 (day 12: Fig. 1C, left panel, PD: PAK 1). The decrease of GTPase levels in PAK 1-depleted lysates quantitatively mirrors the amount of active GTPases (Fig. 1C, Depl: PAK 1) and thus was used for calculating the proportion of active GTPases (Fig. 1D). The percentages of active GTPases in CD40L-stimulated and nonstimulated DCs were calculated from the intensity differences between total and PAK 1-depleted lysates. Results obtained from TL were considered 100% for calculating the values of the corresponding lysates depleted of PAK 1-binding moieties. Mean values (+SD) obtained in three independent experiments, one of which is shown in E. E and F, LPS stimulation augments the activity of Cdc42 and Rac1 in immature, but not mature DCs. Day 10 DCs (E and F) and day 14 DCs that had been stimulated from day 12 to 14 with CD40L (F) were harvested and exposed for 1, 3, or 15 min to LPS. TL and PAK 1-depleted lysates (Depl: PAK 1) were subjected to anti-Cdc42 and anti-Rac1 immunoblotting. No Cdc42 and strikingly reduced Rac1 reactivity were found in PAK 1-depleted samples of artificially activated/GTPγS-loaded lysates (E, last lane), confirming quantitative precipitation of active GTPases. F, The percentages of active GTPases in CD40-stimulated day 14 DCs and nonstimulated day 10 DCs were calculated and plotted as the function of the duration of LPS exposure.

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FIGURE 1. Maturation stimuli increase the activity of Cdc42 and Rac1 in DCs. A and B, DCs up-regulate GTPase expression during development. Day 6, 9, 12, and 14 (±CD40L from day 12 on) cells were harvested and lysed. Equal protein amounts were subjected to SDS-PAGE and immunoblotting with anti-Cdc42 or anti-Rac1 mAbs. Even loading and transfer were controlled by Ponceau red staining of the membranes. B, The ratio of Cdc42 or Rac1 over actin expression is plotted as the function of culture time. C and D, CD40L stimulation augments the activity of Cdc42 and Rac1. DCs were harvested at day 12 and cultured for 24 h in the presence or the absence of CD40L. Lysates of equal cell numbers of untreated or CD40L-stimulated DCs were prepared, and one-half of the samples was used for PD with PAK 1/PBD-coupled agarose. TL, PAK 1-depleted lysates (Depl: PAK 1), and PAK 1-bound proteins (PD: PAK 1) derived from the same number of DCs were separated by SDS-PAGE and immunoblotted. GTPase activity is shown equally by the detection of PAK 1-bound GTPases and, in a more quantitative fashion, by the reduction of anti-GTPase reactivity in the PAK 1-depleted lysates. D, The percentages of active GTPases in CD40L-stimulated and nonstimulated DCs were calculated from the intensity differences between total and PAK 1-depleted lysates. Results obtained from TL were considered 100% for calculating the values of the corresponding lysates depleted of PAK 1-binding moieties. Mean values (+SD) obtained in three independent experiments, one of which is shown in E. E and F, LPS stimulation augments the activity of Cdc42 and Rac1 in immature, but not mature DCs. Day 10 DCs (E and F) and day 14 DCs that had been stimulated from day 12 to 14 with CD40L (F) were harvested and exposed for 1, 3, or 15 min to LPS. TL and PAK 1-depleted lysates (Depl: PAK 1) were subjected to anti-Cdc42 and anti-Rac1 immunoblotting. No Cdc42 and strikingly reduced Rac1 reactivity were found in PAK 1-depleted samples of artificially activated/GTPγS-loaded lysates (E, last lane), confirming quantitative precipitation of active GTPases. F, The percentages of active GTPases in CD40-stimulated day 14 DCs and nonstimulated day 10 DCs were calculated and plotted as the function of the duration of LPS exposure.

LPS stimulation results in the recruitment of Rac1, but not Cdc42, into lipid rafts of DCs

We next explored the mechanisms of GTPase activation in DCs. Lipid raft-associated TLRs have been shown to form a signaling complex with Rac1 in monocytes (35, 36). Thus, we first asked...
whether GTPases are located in lipid rafts or can be recruited into these structures by TLR signaling. Lipid rafts were isolated by sucrose density gradient ultracentrifugation. Proteins of TL and of separated fractions (1–11) were resolved by SDS-PAGE and analyzed by Western blotting. Membranes were probed with mAbs against Cdc42, Rac1, MHC class I, or CD59.

Cdc42 and Rac1 were not detected in rafts of unstimulated DCs. MHC class I H chain was present in raft as well as in nonraft fractions. LPS stimulation led to the rapid recruitment of Rac1 into a subfraction of the lipid rafts in which MHC class I was present in low amounts only (Fig. 2, right panel, lanes 5 and 6). Cdc42 was only detected in the soluble fractions (Fig. 2, lanes 10 and 11).

**FIGURE 2.** LPS stimulation leads to the recruitment of Rac1 into lipid rafts. Untreated (left panel) or LPS-stimulated day 10 DCs (right panel) were lysed and subjected to sucrose density gradient ultracentrifugation. Proteins of TL and of separated fractions (1–11) were resolved by SDS-PAGE and analyzed by Western blotting. Membranes were probed with mAbs against Cdc42, Rac1, MHC class I, or CD59.

**FIGURE 3.** C–D. LPS contact promotes colocalization of Rac1 and GM1. A, Slide-bound day 10 DCs were incubated in the absence (left panel) or presence (right panel) of LPS for 5 min at 37°C. Then DCs were fixed and permeabilized, stained with Ctx B-TRITC (red), anti-Rac1, followed by Alexa Fluor488-conjugated second-step reagent (green), and biotinylated mAb W6/32, followed by SA Alexa Fluor633 (blue), and analyzed by CLSM. CLSM images in A and B show overlays of Rac1 vs GM1 (upper panel) and Rac1 vs MHC class I localization (lower panel). Yellow areas, colocalization of Rac1 and GM1; magenta areas, colocalization of MHC class I and Ctx B. Next to each CLSM overlay image, the respective scatter plots of fluorescence intensity distribution are given. Dots in the upper right quadrants of plots represent pixels that display high intensity for both parameters measured. C, The percentages of GM1$^{high}$ among all Rac1$^{high}$ events, GM1$^{high}$ among all MHC class I$^{high}$ events, and Rac1$^{high}$ among all Rac1$^{high}$ events are given. Data represent mean values (+SD) obtained in five randomly selected microscopic fields covering >30 cell profiles each. Data sets were compared using nonpaired Student’s t test. Note that GM1/Rac1 colocalization increases upon LPS stimulation, while Rac1/MHC class I colocalization remains unaffected. D, Three-color close-up overlay images of typical control (left panel) and LPS-stimulated DCs (right panel) are shown.
We next analyzed these DCs by CLSM for the localization of Rac1, MHC class I, and lipid rafts. Rac1 and the lipid raft marker GM1, a glycosphingolipid detected by Ctx B binding, show virtually nonoverlapping cytoplasmic distribution patterns in unstimulated DCs (Fig. 3, A, upper left panel, and D, left panel). Within 5 min of LPS stimulation, Rac1 and GM1 colocalize (yellow areas in Fig. 3, B, upper left panel, and D, right panel). LPS stimulation also induced a ring-like Rac1 distribution in many cell profiles, indicating stimulation-dependent targeting of this GTPase toward the DC surface (Fig. 3, B and D). On average, 30 ± 8% and 30 ± 9% of the DCs showed clear Rac1-GM1 colocalization at 5 and 15 min after the initial LPS encounter, respectively (Fig. 3B, upper left panel; data not shown). Less than 3% of nonstimulated or mock-stimulated DCs had evidence of Rac1 and GM1 colocalization (Fig. 3A, upper left panel; data not shown). The extent of LPS-induced Rac1-GM-1 colocalization was further quantified in two-dimensional scatter analyses of fluorescence intensity distribution. In nonstimulated DCs, Rac1-high events were mainly GM-1 negative (Fig. 3, A, upper right panel, and C). In LPS-stimulated DCs, 40% of Rac1-high events were also GM-1-high (Fig. 3, B, upper right panel, and C). Evaluation of multiple CLSM images revealed, on average, a 4-fold increase in Rac1-GM-1 colocalization upon LPS stimulation (p < 0.01; Fig. 3C). This value clearly exceeds the 2-fold overall up-regulation of GM-1 reactivity observed upon LPS stimulation (data not shown) and, thus, signifies the specificity of LPS-induced Rac1 targeting into lipid rafts.

Confirming the results of the raft isolation studies (Fig. 2), only a small fraction of Rac1 colocalized with MHC class I, and the extent of colocalization was not modulated after LPS stimulation (Fig. 3, A and B, lower panels, and C). A trend toward increased colocalization of MHC class I and GM-1 was also seen in the scatter analyses (2-fold increased proportion of GM-1-high events in all MHC class I-positive events; Fig. 3C). We can, however, not exclude that this alteration relates to the overall 2-fold increase in GM-1 positivity after LPS stimulation. These results, together with the biochemical results presented in Fig. 2, strongly suggest that the dynamic range of induced lipid raft association is much higher for Rac1 than for MHC class I.

**Perturbation of lipid rafts results in alterations of Cdc42 activity and cytoskeletal organization of DCs**

To further investigate whether GTPases are functionally linked to lipid rafts, we treated DCs with lipid raft-disrupting agents. Incubation of live DCs with MβCD, a substance that destroys lipid rafts by cholesterol extraction, increases the amount of active Cdc42 and, less so, Rac1 (Fig. 4A). Addition of NOG, another lipid raft-disrupting agent, to the lysis buffer similarly augments the levels of PAK 1-binding GTPases in DC lysates (Fig. 4A). The total levels of both GTPases were not changed by NOG treatment or cholesterol extraction (data not shown). Based on the fact that Cdc42 was not found in lipid rafts of DCs (Fig. 2), but is activated by raft-associated receptors and perturbation of lipid rafts, we asked whether purified lipid rafts could activate Cdc42 in vitro. In experiments not shown, we found that the level of active Cdc42 found in the soluble fraction was doubled after mixing and briefly incubating soluble and raft fractions. This suggests that DC rafts indeed bear guanine nucleotide exchange factor (GEF)-like activity for Cdc42. Unlike TLR signaling (Fig. 1, E and F), raft disruption induced Cdc42 activation whether DCs were stimulated by CD40L or not (Fig. 4A). Thus, it appears that Cdc42-directed GEF activity remains associated with lipid rafts irrespective of the maturation stage of the DC.

To analyze whether GTPase activation by perturbation of lipid rafts results in functional responses, we explored the effect of MβCD on the actin-based DC cytoskeleton. As shown in Fig. 4B, MβCD treatment resulted in an immediate effect on the DC actin cytoskeleton. Before MβCD treatment, DCs displayed fine hairy actin protrusions, particularly when cultured in the presence of CD40L (Fig. 4B, upper panel). Within 15 min of exposure to

![FIGURE 4. Perturbation of lipid rafts in DCs results in GTPase activation and remodeling of the DC actin cytoskeleton. A, Cholesterol extraction as well as lipid raft lysis of DCs increase the levels of PAK 1-binding GTPases. Day 12 DCs were incubated in serum-free medium in the presence or absence of MβCD and lysed (upper panel). Day 12 and 14 DCs that had been cultured in the presence of CD40L from day 12 to 14 were lysed in the absence or presence of NOG (lower panel). Lysates of equal cell numbers were used for PD with PAK 1/PBD-coupled agarose. PAK 1-depleted lysates (Depl, right panel) and PAK 1-bound proteins (PD, left panel) were separated by SDS-PAGE and immunoblotted for Cdc42 and Rac1. B, MβCD treatment of DCs results in the rapid induction of F-actin-based filamentous processes. Day 14 DCs cultured in the presence or the absence of CD40L during the last 2 culture days were exposed to MβCD-conditioned or nonconditioned serum-free medium. DCs treated for the indicated time periods, or untreated DCs were fixed, permeabilized, and stained with phalloidin-TRITC. Note the development of arborizing filamentous processes in DCs at early time points. Longer incubation resulted in strongly reduced F-actin contents and, finally, in a loss of the typical DC cytoskeleton (data not shown).](http://www.jimmunol.org/Downloaded)
MβCD, DCs developed a drastic increase in large-sized filamentous processes. Arborizing actin-based surface projections were induced particularly in DCs that were matured in the presence of CD40L before MβCD treatment (Fig. 4B, right panel). These results are compatible with a functionally relevant activation of actin-remodeling GTPases upon lipid raft perturbation. Unlike LPS stimulation, MβCD treatment did not lead to colocalization of Rac1 and GM-1 lipids (W.B., unpublished observations). This may suggest that disruption of rafts dissociates the events of Rac1 localization and activation. The MβCD-induced response occurred in a time-restricted fashion as longer incubation resulted in reduction of processes in numbers and size (Fig. 4B, bottom panel), clumping of actin filaments presumably mediated by disorganized GTPase signaling, and, finally, in severely reduced F-actin content of DCs (data not shown). When MβCD incubation was performed in the presence of free cholesterol, the cells maintained the morphology of nontreated cells (data not shown). These data suggest that lipid rafts can be essential structures for the activation of GTPases, which are critical regulators for the induction and/or maintenance of the actin-based DC morphology.

**DC morphology depends on active Cdc42 and Rac1**

We next tried to identify those GTPases that are critical for the regulation of the actin cytoskeleton of mature DCs. At day 12, we transfected DCs with dominant-negative (N17) mutants of Cdc42 and Rac1 (Fig. 5A). A bicistronic vector encoding myc-tagged mutant proteins and EGFP was used. Transfected cells were matured for 2 days in the presence of CD40L and then analyzed by CLSM. The intracellular localization of the individual transfected GTPases was visualized in EGFP+ cells by anti-myc staining. Distribution of F-actin was visualized by staining with TRITC-coupled phalloidin (Fig. 5A). Actin filaments of mock-transfected DCs are connected to the cell surface and extend into spike-like protrusions (Fig. 5A, panel 1). DCs transfected with N17 mutants become essentially round and show a dramatic reorganization of the cytoskeleton (Fig. 5A, panels 2 and 3). This morphological alteration was more pronounced in the N17Cdc42- DCs that lose virtually all actin spikes. N17Rac1- transfectants still form small actin protrusions, although much reduced in number. Frequently, N17Rac1+ DCs appeared to be surrounded by a thin rim of membrane with the appearance of a collapsed lamellipodium (Fig. 5A, panel 3). These aspects suggest a Cdc42-independent function of Rac1 in the formation of the typical DC cytoskeleton. This is further supported by our observation that reduced F-actin levels and DC morphology in N17Rac1+ DCs cannot be restored by cotransfection of constitutively active L61 mutants of Cdc42 (data not shown). Of note is the further observation that N17Cdc42- and N17Rac1-transfected DCs failed to accumulate filamentous processes in response to raft disruption (data not shown), indicating that these GTPases are also critical regulators in the former assay.

We asked whether L61GTPase mutants induce a DC-like morphology in nondendritic APCs. Mφ were differentiated from CD34+ HSCs in M-CSF/IL-6-conditioned medium. In mock-transfected Mφ actin filaments are modestly structured in few, rather stout protrusions (Fig. 5B, panel 1). L61Cdc42-transfected Mφ up-regulate F-actin levels (Fig. 5B, panel 2) just like DCs during their differentiation from the same progenitors. More importantly, the distribution of F-actin in L61Cdc42+ Mφ resembles closely that observed in control DCs (compare Fig. 5A, panel 1, and Fig. 5B, panel 2). L61Rac1+ Mφ acquired a fried-egg-like phenotype (Fig. 5B, panel 3) and were surrounded by a giant lamellipodium that contained several radial actin spikes. Thus, active Cdc42 seems to be a key element for controlling the formation and maintenance of the F-actin-based DC cytoskeleton. The contribution of Rac1 is less prominent. Nevertheless, active Rac1 is required to maintain full DC morphology.

**GTPases control surface expression of DC maturation-related immunostimulatory molecules**

Turley et al. (37) showed previously that LPS induced the actin-dependent export of MHC class II-peptide complexes in murine DCs. In this study, we analyzed the effect of the F-actin inhibitor Lat B on the LPS-induced surface expression of MHC class I and II and CD86. Time kinetics confirmed that Lat B suppressed not

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**FIGURE 5.** Cdc42 and Rac1 activity regulates DC morphology. A, CD34+ HSC-derived DCs, transfected with vector control, N17Cdc42, or N17Rac1 at day 12, were cultured in the presence of CD40L until day 14. B, Mφ were generated from CD34+ HSCs by culture in M-CSF/IL-6-conditioned medium. Mφ were transfected with vector control, L61Cdc42, or L61Rac1 at day 12 of their development and cultured for 1 more day in M-CSF/IL-6-conditioned medium. Cells were fixed, permeabilized, and incubated with phalloidin/TRITC (red) and anti-myc, followed by Alexa Fluor633-conjugated second-step reagent (blue). Productively transfected cells within bulk DC populations were identified by EGFP fluorescence (green) and F-actin and myc-tagged GTPase expression was recorded in multiple cell profiles. N17Cdc42+ and N17Rac1+ DCs lost their dendritic shape and became essentially round. L61Cdc42+ Mφ display a DC-like morphology, and L61Rac1+ induced lamellipodia formation in Mφ. CLSM images shown are representative of three independent experiments. Virtually identical phalloidin-staining results were obtained when DCs were first sorted according to EGFP and CD1a expression and then were analyzed by CLSM (data not shown).

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only the export of MHC class II, but also of MHC class I and CD86 (Fig. 6A). Expectedly, BFA strongly blocked the export of these molecules from the Golgi to the plasma membrane (Fig. 6A).

These data together with our observation that GTPases regulate actin polymerization in DCs (Fig. 5A) imply that Cdc42 and Rac1 control actin-dependent transport of immunostimulatory molecules. Thus, we analyzed surface expression of these molecules in N17GTPase-transfected DCs before and after CD40L stimulation. FACS experiments showed a reduced CD86 surface expression in N17Cdc42− DCs and a significant, but less pronounced, reduction in N17Rac1+ transfectants (Fig. 6B). Compared with mock-transfected cells, W6/32 FACS analysis depicts a significant reduction of surface MHC class I in N17Cdc42− and in N17Rac1+ DCs (Fig. 6C). This deficit in CD86 and MHC class I surface expression could not be overcome by stimulation of DCs with CD40L.

These experiments also show the diminished surface expression of GM1 glycosphingolipid in both transfectants after CD40L stimulation (Fig. 6E). In contrast, CD40L stimulation compensated the slightly reduced levels of MHC class II molecules in N17 transfectants (Fig. 6D). IL-12 secretion, which is also up-regulated in CD86 secretion, which is also up-regulated in CD40L-matured DCs, was not altered by dominant-negative GTPases (Fig. 6F). Likewise, CD40L-induced NF-κB and JNK activation proceeded in unperturbed fashion in N17Rac1 and in N17Cdc42-transfected DCs (E.K., unpublished observations). Thus, these data suggest that GTPases specifically regulate the expression of CD86 and MHC class I molecules and lipid rafts.

To further investigate the possibility that GTPases regulate protein export, the transport of conformationally intact MHC class I to the cell surface was studied by FACS. After acidic elution of mAb W6/32-defined conformationally intact MHC class I/β2-m/peptide complexes, the accumulation of new W6/32-reactive moieties on the surface was clearly reduced in N17GTPase-transfected DCs as compared with control-transfected DCs (Fig. 6G). These experiments suggest that GTPase activity is involved in the regulation of the export of molecules that determine the immunostimulatory capacity of DCs.

**Active GTPases regulate the maturation-dependent stimulatory potential of DCs for CD8+ T cell proliferation**

To test the stimulatory potential of differently matured DCs, we performed allogeneic MLRs with unstimulated and CD40L- or LPS-stimulated DCs. DCs, matured in the presence of LPS or...
CD40L, up-regulate levels of CD86 and MHC molecules (4, 34) (Fig. 6A). Purified naive CD4+ or CD8+ T cells were used as responders. Proliferation increased 3.5- and 4.2-fold when CD4+ T cells were cocultured with CD40L- and LPS-treated DCs as compared with control DCs, respectively (Fig. 7A). Similarly, DCs tripled their stimulatory potential for CD8+ T cells upon stimulation with CD40L or LPS (Fig. 7A). So far, our data indicate that maturation signals control GTPase activity that itself controls the display of immunostimulatory molecules. Is the GTPase activity in DCs of relevance for the occurrence and magnitude of proliferative T cell responses? Allogeneic MLRs were performed with transfected CD40L-stimulated DCs and cord blood-derived naive CD4+ or CD8+ T cells (Fig. 7, B and C). Mock- as well as N17GTPase-transfected DCs expressing EGFP at least 1.5 log above the background fluorescence level were sorted. This calibration was used because N17GTPase+ DCs expressing EGFP above this value were strongly impaired in their ability to chemo-tactically respond to chemokine ligands, a response dependent on GTPase function (7) (data not shown). The MLR stimulatory and migratory capacity of mock-transfected, sorted GTPase+ DCs was comparable to those of nontransfected control DCs (data not shown). Fig. 7B shows that all transfectants were perfectly able to induce allogeneic CD4+ T cell responses. These data correlate with our observation that the slight reduction of surface MHC class II levels was equated by CD40L stimulation (Fig. 6D). In contrast, induction of CD8+ T cell proliferation by both N17GTPase-transfected DCs was significantly impaired (Fig. 7C). CD8+ T cell proliferation induced by GTPase-deficient CD40L-activated DCs was reduced to or below the level induced by nonactivated DCs. The effect of N17Cdc42 was more pronounced than that of N17Rac1 transfection. Thus, Cdc42 and Rac in DCs are important for the induction of CD8+ T cell proliferation, but dispensable for the occurrence of Th cell activation.

**Active GTPases regulate the delivery of costimulatory signals for CD8+ T cell proliferation**

To further classify the functional defect in CD8+ T cell induction, we performed costimulation assays. CD40L-stimulated N17Cdc42+ DCs and mock-transfected DCs were cocultured with autologous naive CD8+ T cells. The level of TCR triggering in this assay was adjusted by defined amounts of immobilized anti-CD3 mAbs (Fig. 7D). We detected a strong costimulatory activity of control DCs for naive CD8+ T cells when >0.3 μg/ml anti-CD3 mAbs were used for coating (Fig. 7D, □). Under the same conditions, N17Cdc42+ DCs largely failed to induce T cell stimulation (Fig. 7D, □). We thus conclude that GTPase signaling in DCs is essential for the delivery of costimulatory signals to CD8+ T cells, and that the consequences of GTPase deficiency in DCs cannot be overcome by delivering of a potent TCR signal. In experiments not shown, we also titrated graded amounts of cross-linked anti-CD28 mAbs to allogeneic MLRs in which N17GTPase- and control-transfected DCs were used as stimulators for CD8+ T cells. Anti-CD28 mAbs increased the weak T cell response induced by N17GTPase-transfected DCs, but could not fully reconstitute the

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**FIGURE 7.** GTPases regulate DC maturation-dependent CD8+ T cell responses. A, Activated DCs are effective stimulators of CD8+ and CD4+ T cells. Day 10 DCs were harvested and cultured in the presence of LPS (■), CD40L (□), or medium only (□) until day 13. A total of 1 x 10^5 DCs was then cocultured with 5 x 10^4 naive allogeneic CD4+ or CD8+ T cells. B and C, DCs require active Cdc42 and Rac1 to induce strong CD8+ T cell proliferation. DCs were transfected at day 12 with the indicated constructs, and sorted at day 13 as EGFP+/CD1a+ cells (purity >95%). Sorted cells were cultured for 2 more days in CD40L-conditioned medium. Graded numbers of DCs (B and C, x-axis), transfected with vector control ( ), N17Rac1 (△), or N17Cdc42 (○), were cocultured with 5 x 10^4 naive CD4+ (B) or CD8+ (C) T cells. D, N17Cdc42+ DCs lack costimulatory activity for CD8+ T cells. A total of 5 x 10^4 naive CD8+ T cells was cultured in 96-well round-bottom plates coated with graded concentrations of anti-CD3 mAbs (μg/ml, x-axis) in the absence (□) or the presence of flow-sorted, autologous CD40L-stimulated, control (□)- or N17Cdc42 (○)-transfected DCs (1 x 10^4). E and F, Overexpression of activated Cdc42 and Rac1 in Mφ does not increase their poor T cell stimulatory activity. Mφs were transfected at day 12 with the indicated constructs, and sorted at day 13 as EGFP+/CD4+ cells (purity >90%). Graded numbers of Mφ (E and F, horizontal axis), transfected with vector control ( ), L61Rac1 (△), and L61Cdc42 (○), were cocultured with 5 x 10^4 naive CD8+ (E) or CD4+ (F) T cells. Cells were pulsed with [3H]thymidine during the last 16 h of the 6-day (A-C, E, and F) or 4-day (D) MLR. T cell proliferation was assessed by measuring the [3H]thymidine uptake by liquid scintillation counting. Results are expressed as mean cpm ± SD (y-axis). Results are representative of three independent experiments.
level of proliferation induced by control-transfected DCs. Thus, it appears that the weak stimulatory capacity of N17GTPase-transfected DCs is, at least, due to a deficit in the delivery of costimulatory signals.

The GTPase-driven induction of filamentous surface projections in Mφ has no augmentative function for their T cell stimulatory capacity

Based on the observation that L61GTPases induced DC morphology in Mφ (Fig. 5B), we investigated whether active GTIPases can change the T cell stimulatory properties of these nonprofessional APCs. The allostimulatory activity of Mφ was not boosted by L61GTPase transfections (Fig. 7, E and F). Thus, a dendritic surface configuration of an APC per se is not directly linked to strong immunostimulatory capacity. In summary, our data suggest that LPS- or CD40L-mediated maturation signaling acts via a GTPase-dependent pathway to up-regulate costimulatory mechanisms, and consequently to induce efficient CD8\(^+\) T cell proliferation.

Discussion

This study assigns novel functions to the GTIPases Cdc42 and Rac1 in the signaling cascades inducing phenotypic and functional maturation of human DCs. As previously reported, substantial amounts of Cdc42 and Rac1 are active in resting DCs. More importantly, we show that: 1) GTPase activity is up-regulated by signals through receptors of the innate as well as the adaptive immune system (i.e., LPS receptors and CD40); 2) Cdc42 and Rac1 activity is required for the induction and maintenance of the typical cytoskeletal architecture of mature DCs; and 3) those GTIPase activities apparently also control the immunostimulatory program that is responsible for the elicitation of CD8\(^+\) T cell responses.

The previous demonstration of a critical role of Cdc42 and Rac1 in constitutive macropinocytosis shows the involvement of active GTIPases in a cell function restricted to certain immature DCs (13, 14). Ig receptor-mediated Ag uptake, another function of immature DCs, is likewise dependent on Cdc42 and Rac1 in Mφ (38). As shown in this study, mature human DCs, in contrast to mouse bone marrow DCs (13), contain both active Cdc42 and active Rac1. Functionally, mature human DCs require both GTIPases for the maintenance of the typical DC actin cytoskeleton as well as for the proper display of MHC and costimulatory molecules. The functional roles of these GTIPases in DCs thus extend beyond Ag uptake. It is also a conclusion of this study that the type of biologic responses that Cdc42 and Rac1 regulate must depend on the differentiation stage of the DCs. Hence, immature and mature DCs must fundamentally differ in the expression and/or the distribution of the target molecules of GTIPases that translate GTPase activation into biological responses.

Cdc42 and Rac1 exhibit similar, but not identical functions for DCs. This is shown by the different effects of transfected GTIPase mutants on the morphology of mature DCs. DCs depleted of functional Cdc42 were devoid of filopodia and lamellipodia, while Rac1-deficient DCs lose their lamellipodia, but still have poorly developed filopodia. Expression of activated Cdc42 could not compensate for the morphologic deficit imposed by dominant-negative Rac1 and vice versa. These data suggest that Rac1 and Cdc42 activity in DCs can be rather selectively eliminated by induced expression of the respective dominant-negative mutants. Moreover, it follows that Rac1 and Cdc42 regulate two rather separate signal transduction pathways in the assembly of the cell lineage-defining filamentous structures of DCs.

The mode of activation of the two GTIPases also varies considerably. Rac1 is instantaneously recruited to lipid rafts during LPS-induced DC activation. The cytosolic domain of LPS-triggered TLR may be the docking and activation site for Rac1 within the rafts, as previously suggested in studies using monocytic cell lines (35). Cdc42 is not targeted to DC rafts, although activated by LPS with similar kinetics as Rac1. In line, Cdc42 does not bind to TLR (35). Cdc42 activation may still be accomplished by raft-dependent signals. We detected a GEF-like activity for Cdc42 in lipid raft fractions from DCs. Candidate molecules for this activity include Vav, members of the family of diffuse B cell lymphoma (Dbi) oncogene-related and -CED-5/DOCK180/myoblastosis (CDM) proteins (39–41), and phosphoinositides. Phosphatidylinositol-4,5-bisphosphate is highly enriched in rafts and has GEF activity for Cdc42 (42, 43). Thus, raft components may initiate and regulate both Cdc42 and Rac1 activation also in DCs. Further arguments for this hypothesis are derived from our observation that the disruption of lipid rafts results in pronounced GTPase activation and, perhaps as the consequence, in the modulation of the DC actin cytoskeleton. As previously suggested for fibroblasts (20), lipid rafts are thus apparently signaling platforms for GTPase-dependent actin rearrangements in human DCs. The importance of these GTIPases most likely extends their role in transducing signals initiated by raft-associated receptors. Cdc42 and Rac1 regulate the surface representation of lipid microdomains in DCs, a mechanism previously noted in mast cells as well (19). It thus appears that Cdc42 and Rac1 are key players in the positive feedback loop that regulates the constitutive and activation-induced display and function of rafts and of their associated signaling receptors.

The localization of MHC class I in rafts might appear surprising in view of contradictory data obtained with Mφ and monocytes (44). In B cells, however, MHC class I was found in microdomains and vesicles that are frequently juxtaposed to lipid rafts (45). Our study unambiguously demonstrates the presence of MHC class I in lipid rafts of DCs by independent biochemical approaches as well as microscopy. The observation of Rac1 and MHC class I recruitment to largely nonoverlapping populations of surface rafts suggests that functionally specialized sets of lipid rafts exist on DCs. The Rac1-targeted rafts may be the platforms of outside-in signaling, while MHC class I-containing rafts may be specialized for optimized Ag display.

LPS stimulation induced the actin-dependent recruitment of MHC class I as well as of costimulatory molecules to the DC surface. Blockade of GTPase activity induced a significant reduction in the stimulation-induced transport of these molecules to the cell surface. The molecular details such as the exact point(s) of blockade and the extent to which this pathway depends on actin assembly remain to be established. Actin-dependent and -independent functions of Cdc42 in protein export and sorting are firmly established (16–18). Evidence for Rac-dependent protein export comes from the studies showing that Rac1 activated by the Ewing’s sarcoma Ag CD99/Mic2 is critical for MHC class I export in B cells. In CD99-deficient cells, MHC class I molecules are synthesized and assembled normally, but are trapped at the level of the post-Golgi (46). This export defect can be rescued by activated Rac1 (47). Thus, Golgi export of MHC class I in DCs may likewise be regulated by GTIPases. Importantly, Rac1 and Cdc42 are not required for all protein export functions in DCs, nor do dominant-negative GTIPase induce an overall signaling defect in DCs. Well-established maturation-dependent DC functions, like IL-12 secretion after CD40 ligation (34) and the activation of certain biologically important intracellular signaling pathways, remain unperturbed. Expression of surface MHC class II, known to be enriched in lipid rafts and tetraspan microdomains (48), was only
slightly reduced by dominant-negative mutants. Thus, it appears that only a selective set of molecules is regulated by this pathway that may be needed to accomplish defined cell functions.

A DC function found to depend on GTPase activity is the activation of CD8+ T cells. We were able to demonstrate that CD40L-matured, N17GTase-transfected DCs elicit efficient CD4+ T cells, but failed to induce strong CD8+ T cell responses. This inhibitory effect of N17Cdc42 and N17Rac1 could result from the reduced surface expression of MHC class I and costimulatory molecules, morphologic alterations, or a combination of both. Impaired MHC expression alone unlikely is the major factor determining this stimulatory deficit because we show that the impaired CD8+ T cell stimulatory capacity of GTase-deficient DCs cannot be overcome by saturated TCR triggering. Thus, GTPase signaling should be of relevance for the maturation-dependent display of costimulatory molecules, as exemplified for CD86, or for other costimulation-promoting mechanisms that may include the cells’ ability to efficiently contact T cells due to their particular surface architecture. Although our experiments with L61GTase-transfected M6 show that the poor allostimulatory capacity of these cells remains unaltered when these cells are manipulated to display DC-like morphology, it remains possible that an APC surface covered by filamentous processes becomes important for T cell activation only in the context of a true DC.

In summary, the GTases Cdc42 and Rac1 have essential functions at various biologically important stages during the life cycle of DCs. They regulate Ag uptake in immature DCs; they participate in the transduction of signals that lead to maturation; and, in mature DCs, they regulate cell shape and immunostimulatory functions. It remains to be determined in detail how these GTPases manage to mediate these diverse events and what is the nature of the apparently distinct downstream effectors that finally mediate these biologic functions.

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