

Cdc42 and RhoB Activation Are Required for Mannose Receptor-mediated Phagocytosis by Human Alveolar Macrophages

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Human alveolar macrophages (AMs) phagocytose *Pneumocystis* (*Pc*) organisms predominantly through mannose receptors, although the molecular mechanism mediating this opsonin-independent process is not known. In this study, using AMs from healthy individuals, *Pc* phagocytosis was associated with focal F-actin polymerization and Cdc42, Rac1, and Rho activation in a time-dependent manner. Phagocytosis was primarily dependent on Cdc42 and RhoB activation (as determined by AM transfection with Cdc42 and RhoB dominant-negative alleles) and mediated predominantly through mannose receptors (as determined by siRNA gene silencing of AM mannose receptors). *Pc* also promoted PAK-1 phosphorylation, which was also dependent on RhoGTPase activation. HIV infection of AMs (as a model for reduced mannose receptor expression and function) was associated with impaired F-actin polymerization, reduced Cdc42 and Rho activation, and markedly reduced PAK-1 phosphorylation in response to *Pc* organisms. In healthy AMs, *Pc* phagocytosis was partially dependent on PAK activation, but dependent on the Rho effector molecule ROCK. These data provide a molecular mechanism for AM mannose receptor-mediated phagocytosis of unopsonized *Pc* organisms that appears distinct from opsonin-dependent phagocytic receptors. Reduced AM mannose receptor-mediated Cdc42 and Rho activation in the context of HIV infection may represent a mechanism that contributes to the pathogenesis of opportunistic pneumonia.

INTRODUCTION

Opportunistic lung infections such as *Pneumocystis* pneumonia frequently complicate HIV disease, although the underlying mechanisms of increased susceptibility and disease pathogenesis remain incompletely understood. Alveolar macrophages (AMs) are critical components of the pulmonary innate immune response to infectious challenge and likely represent important effector cells in the host response to *Pneumocystis* (Limper *et al.*, 1997; Steele *et al.*, 2003). Human AMs express surface innate immune receptors such as mannose receptors (Greenberg and Grinstein, 2002) that can mediate *Pneumocystis* phagocytosis (Ezekowitz *et al.*, 1991). AMs from asymptomatic HIV-infected individuals at high clinical risk for *Pneumocystis* pneumonia (peripheral CD4⁺ T-lymphocyte counts <200 cells/mm³) demonstrate significantly reduced *Pneumocystis* phagocytosis compared with AMs from healthy individuals (Koziel *et al.*, 1998a). Al-

though the reduced *Pneumocystis* phagocytosis is in part associated with reduced AM mannose receptor surface expression (Koziel *et al.*, 1998a), the mechanism of reduced phagocytosis has not been completely established.

The family of small Rho guanosine triphosphatases (GTPases) link membrane receptors to the actin cytoskeleton and represent molecular switches that control a variety of signal transduction pathways including phagocytosis (Etienne-Manneville and Hall, 2002). Rho GTPases are a subgroup of the Ras superfamily of 20–30-kDa GTP-binding proteins, and include Cdc42, Rac, and Rho (-A, -B, and -C). Specific Rho GTPase activation may provide the molecular basis for the observed differences in opsonin-dependent receptor-mediated phagocytosis described for immune globulin receptors (FcγR) and complement receptors (C3R). FcγR-mediated phagocytosis of appropriately opsonized particles may be mediated predominantly by Cdc42 and Rac, whereas C3R-mediated phagocytosis of appropriately opsonized particles may be mediated primarily by Rho (Caron and Hall, 1998). However, the role of the actin-based cytoskeleton and the molecular mechanisms that mediate opsonin-independent phagocytosis of *Pneumocystis* by human AMs have not been investigated. The purpose of this study was to examine the role of RhoGTPases in opsonin-independent receptor-mediated phagocytosis of *Pneumocystis* organisms by human AMs and to examine the influence of HIV infection on

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Abbreviations used: GTPases, guanosine triphosphatases; HIV, human immunodeficiency virus; PAK-1, p21-activated kinases; ROCK, Rho-associated coiled-coil kinase.

Pneumocystis-mediated AM RhoGTPase expression and activation.

MATERIALS AND METHODS

Study Subjects

Prospectively recruited healthy and asymptomatic HIV seropositive (HIV+) individuals were without evidence for active pulmonary disease and had normal spirometry. Healthy individuals were without known risk factors for HIV infection and confirmed to be HIV seronegative by ELISA (ELISA performed according to the manufacturers instructions; Abbott Diagnostics; N. Chicago, IL). Demographic characteristics for all participants included age, gender, smoking status, HIV risk factor, prior opportunistic infections, and prescribed antiretroviral medications.

Bronchoscopy

Pulmonary immune cells were obtained by bronchoalveolar lavage (BAL) using standard technique as previously described (Koziel *et al.*, 1998a, 1998b). All procedures are performed on consenting adults following protocols approved by the Beth Israel Deaconess Medical Center institutional review board and Committee for Clinical Investigations. The cells were separated from the pooled BAL fluid as previously described (Koziel *et al.*, 1998a) and counted on a hemacytometer with light microscopy.

Human AMs

AMs were isolated by adherence to plastic-bottom tissue culture plates ($1-3 \times 10^6$ cells/well in six-well plates for Western blot, transfection and immunoprecipitation) or 13-mm round glass coverslips ($1-2.5 \times 10^5$ cells/slip in 24-well plates for phagocytosis and immunofluorescence staining) as previously described (Koziel *et al.*, 1998a). Isolation of AMs from all healthy and HIV+ persons yielded cells that were $\geq 98\%$ viable as determined by trypan blue dye exclusion and demonstrated $>95\%$ positive nonspecific esterase staining.

In Vitro HIV-1 Infection of AMs from Healthy Individuals

To assess the direct effect of HIV-1 infection on macrophage RhoGTPase activation, AMs from healthy individuals were infected in vitro as previously described (Koziel *et al.*, 1998a; Salahuddin *et al.*, 1986), utilizing a monocytotropic (R5) HIV-1 isolate (HIV Bal). Uninfected macrophages were also maintained as control conditions. Culture media was changed every 3-4 d, and productive HIV-1 infection was verified by the measurement of HIV p24 antigen in the culture supernatants by ELISA (Dupont, Boston, MA). At 2 wk after HIV-1 infection, viability was determined on representative cells by

trypan blue dye exclusion, and experiments were performed comparing HIV-1-infected to -uninfected AMs.

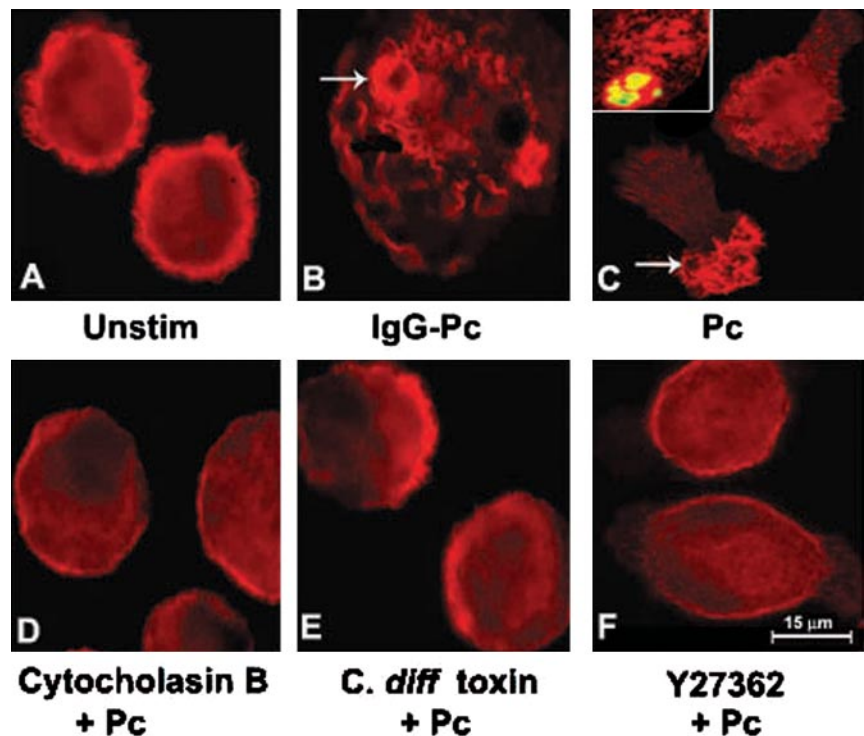
AM Innate Immune Receptor Stimulation

To examine the role of AM Rho GTPase signaling pathway during opsonin-independent phagocytosis, experiments used PMA (phorbol myristate acetate; Sigma Chemical, St. Louis, MO), zymosan (Sigma Chemical) and *Pneumocystis*. PMA is a recognized inducer of Rho GTPase in neutrophils and served as a positive control. Unopsonized zymosan and *Pneumocystis* are recognized by macrophage innate immune receptors (Ezekowitz *et al.*, 1991; Hoffmann *et al.*, 1999). Because sustainable cultivation of *Pneumocystis* is not possible, and *Pneumocystis* derived from human disease (*P. jiriveci*; Stringer *et al.*, 2002) is rarely available, *Pneumocystis* organisms were obtained from chronically immunosuppressed male Lewis rats (University of Cincinnati Lab Animal Medicine Facility, Cincinnati, OH) as previously described (Zhang *et al.*, 2004). Isolated *Pneumocystis* mixed-life cycle preparations yield $\sim 90\%$ trophozoite and 10% cyst forms, and viability was $>85\%$ (Chen and Cushion, 1994). *Pneumocystis* preparations were relatively free of contaminating rat-derived proteins (Koziel *et al.*, 1998b), and preparations were endotoxin-free (<1.0 endotoxin units/ml) as determined by E-toxate *Limulus polyphemus* assay (Sigma Chemical).

Immunofluorescence Microscopy

To demonstrate intracellular localization of F-actin, Cdc42, Rac1, Rho, and β -tubulin, isolated AMs (2×10^5 cells/coverslip) were rinsed twice with cytoskeletal buffer, fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in phosphate-buffered saline (PBS) for 10 min at room temperature (RT), and permeabilized with 2% triton in 1% bovine serum albumin (BSA) in PBS, and then incubated with unconjugated primary antibodies for 1 h at 37°C. Primary antibodies included the following: anti-Cdc42, anti-Rac1, anti-RhoB (Santa Cruz Biotechnology, Santa Cruz, CA), anti- β -tubulin and anti- β -actin (Sigma-Aldrich). After washing with PBS, cells were incubated a goat anti-rabbit secondary antibody conjugated either with Cy3 (Sigma Chemical) or a goat anti-mouse secondary antibody conjugated with fluorescein isothiocyanate (FITC; Sigma Chemical) for 1 h at 37°C. Control conditions included cells stained with primary or secondary antibody only. For select experiments cells are counterstained with the nucleic acid stain DAPI (1:500; Molecular Probes, Eugene, OR; 1:500) for 30 min at RT. For detection of F-actin polymerization, cells were stained with Rhodamine-phalloidin (Molecular Probes). After washing five times with PBS, coverslips were mounted with Mowiol 4-88 (Hoechst Celanese, Somerville, NJ), and images were captured using a fluorescence microscope equipped with a digital camera (Nikon Eclipse E800; Diagnostic Instruments; Sterling Heights, MI).

Figure 1. Focal F-actin polymerization during *Pneumocystis* phagocytosis by AMs from healthy individuals. Immunofluorescence microscopy images of isolated AMs after incubation with *Pneumocystis* organisms (ratio 10:1 organisms to macrophages) for 20 min at 37°C in 5% CO₂. F-actin was detected by rhodamine-phalloidin (red color) as described in *Materials and Methods*. (A) Unstimulated AMs demonstrate cortical distribution of F-actin. (B) Focal accumulation of F-actin (i.e., phagocytic cup) was evident in AMs at points of contact with IgG-opsonized *Pneumocystis* (IgG-Pc; positive control for F-actin polymerization; arrow). (C) Focal accumulation of F-actin was evident in AMs at points of contact with unopsonized *Pneumocystis* (Pc; arrow). Inset demonstrates F-actin polymerization on the macrophage surface surrounding FITC-labeled *Pneumocystis* organism (yellow color). No F-actin polymerization was observed in the presence of cytochalasin B (D; an inhibitor of actin polymerization), *C. difficile* toxin B (E; a general inhibitor of small GTPases), or Y27632 (F; a specific inhibitor of Rho kinase, Rock). Depicted images from one healthy individual are representative of AMs from at least four individuals examined.



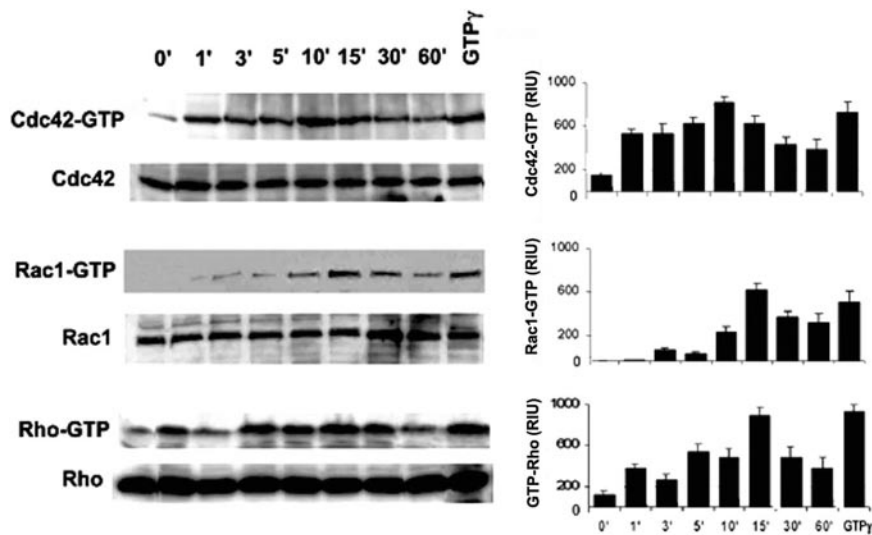


Figure 2. *Pneumocystis* activates Cdc42, Rac1, and Rho in AMs from healthy individuals. AMs are incubated with unopsonized *Pneumocystis* organisms (10:1) up to 60 min at 37°C in 5% CO₂, and Western blot on cell lysates was performed to detect both total and activated (–GTP) form of Cdc42, Rac1, and Rho. The lane for GTP- γ is a positive control for the assay. Western blots are shown for AMs from one healthy individual and are representative of experiments from three different individuals. The corresponding bar graphs represent densitometry quantitative analysis for Cdc42-GTP, Rac1-GTP, and Rho-GTP (n = 3) expressed as relative intensity units (RIU).

Western Blot

Western blot was performed as previously described (Zhang *et al.*, 2004) using polyclonal anti-Cdc42, anti-Rac, anti-RhoB and anti-PAK, phospho-PAK, and monoclonal anti-Rho A (Santa Cruz Biotechnology). After blocking with 4% nonfat dry milk, membranes were incubated with appropriate antibodies overnight at 4°C, washed three times with TTBS, incubated with conjugated secondary antibody (1:3000) for 2 h, and washed three times with TTBS, and then the signal was detected by SuperSignal West Pico protein detection system (Pierce, Rockford, IL). Protein concentrations were determined by Bio-Rad protein assay using BSA as standard (Bio-Rad, Hercules, CA). Select experiments used the general Rho GTPase inhibitor, *Clostridium difficile* toxin B (Sigma) or the specific Rho Kinase inhibitor, (+)-R-trans-4-(aminoethyl)-N-4-pyridyl) cyclohexanecarboxamide (Y-27632; Calbiochem, San Diego, CA; Uehata *et al.*, 1997).

Cdc42, Rac, and Rho Activation Assay

AM cytoplasmic extracts were prepared, and activation of Cdc42, Rac (Benard *et al.*, 1999), and Rho (Ren *et al.*, 1999) were determined by affinity precipitation using GST fusion-protein-based kits according to the manufacturer's protocols (Rac1/Cdc42 assay reagent using GST-PBD, and Rhotekin Rho binding domain using GST-RBD; Upstate Biotechnology, Lake Placid, NY). For Cdc42 and Rac1-GTP, proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and blotted for the appropriate specific monoclonal antibodies (Santa Cruz Biotechnology). To identify Rho-GTP, immobilized Rhotekin (Rho binding domain) was used to selectively precipitate Rho-GTP (Upstate Biotechnology), and precipitated GTP-Rho was detected by immunoblot using polyclonal antibody (-A, -B and -C; Upstate Biotechnology). For all assays, signal was detected by SuperSignal West Pico protein detection system (Pierce).

Dominant Negative Cdc42, Rac, and Rho Allele Amplification, Purification, and Transfection

Guanine nucleotide binding-deficient (dominant negative) N-terminal 3 \times hemagglutinin (HA)-tagged alleles Cdc42 T 17 N, Rac1 T17 N, Rho A T19 N, and Rho B T19 N subcloned in pcDNA 3.1+ vector (Guthrie cDNA Resource Center, Sayre, PA) were used for transfections. The empty vector pcDNA 3.1+ (Invitrogen, Carlsbad, CA) was used as a control. Plasmid amplification was performed using MAX Efficiency DH5 α MAX Competent *Escherichia coli* (Invitrogen) as previously described (Miralem and Avraham, 2003). DNA purification was performed using Geno Pure Plasmid Maxi Kit (Roche, Indianapolis, IN) following the manufacturer's protocol, and cDNA was confirmed by resolution on agarose gel and optical density measurement (260 nm) by spectrophotometry. Transfection of AMs (1 \times 10⁶ cells/well of six-well plate) of each dominant negative allele was performed using Effectene Transfection Reagent (Qiagen, Valencia, CA) for 60 h.

Immunoprecipitation

Transfected AM cell cytoplasmic protein was prepared using NE-PER kit (Pierce) according to the manufacturer's protocol. N-terminal 3 \times HA-tagged dominant negative Rho GTPase proteins were immunoprecipitated by incubation cytoplasmic protein with monoclonal antibody against 3 \times HA for overnight at 4°C and then the antibody-protein complexes were incubated

with protein A-agarose (Upstate Biotechnology) for 2 h at RT. Each immunoprecipitate was washed three times with PBST. The agarose beads and protein complex were solved by SDS-PAGE as described above, and immunoblot was probed for 3 \times HA.

Fluorescein Isothiocyanate Labeling of *Pneumocystis*

To facilitate the identification of *Pneumocystis* in macrophage phagocytosis studies, organisms were labeled with FITC (Sigma Chemical), as previously described (Ezekowitz *et al.*, 1991). The FITC-labeling process stained both trophic and cyst form surfaces uniformly and did not affect organism viability as determined by flow cytometry (Armstrong *et al.*, 1991).

AM Phagocytosis of *Pneumocystis*

This technique allowed for the discrimination of FITC-labeled *Pneumocystis* phagocytosed by the macrophages from those only bound to the surface. AMs adherent to round glass cover slips were incubated with FITC-labeled *Pneumocystis* at an organism-to-cell ratio of 5:1 for 60 min at 37°C in 5% CO₂. After washing with Hanks' balanced salt solution containing magnesium and calcium (Hanks' balanced salt solution+), the AM monolayers were fixed in 4% paraformaldehyde overnight at 4°C, and confocal microscopy was used to determine the phagocytic and binding index as previously described (Koziel *et al.*, 1998a), using a Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA) fitted with a 25-mW argon-ion laser. Experimental conditions were performed in duplicate, and the number of FITC-labeled organisms phagocytosed per 200 macrophages was counted on at least two separate slides.

siRNA Gene Silencing of AM Mannose Receptors and PAK1

To determine the specific contribution of AM mannose receptors to *Pneumocystis*-mediated RhoGTPase signaling and to determine the role of PAK1 in *Pneumocystis* phagocytosis, siRNA gene silencing was used to produce functional "knock-down" of human AM mannose receptors or PAK1 as previously described (Zhang *et al.*, 2004). Experiments utilized the following oligonucleotide (annealed double-stranded siRNA; Qiagen): Human mannose receptor siRNA (siRNA-MR) sequences: DNA target sequences: AAGTGG-TACGCAGATTGCACG begin from 528 base pairs to 549 base pairs; 5'-GUGGUACGCAGAUUGCACG-3' and 3'-CGUGCAAUCUGCGUACCAC-5'. Human PAK1 siRNA (siRNA-PAK1) sequences: DNA target sequences: 5'-AAGGAGAAGAAAAAGAAGGAC-3'. Sense: 5'-GGAGAAGAAAA-GAAGGACtt-3'. Antisense: 5'-GUCCUUCUUUUUCUUCUCctc-3'. MR siRNA and PAK1 siRNA were individually transfected into AMs using TransMessenger Transfection reagent (Qiagen).

Detailed procedures were performed following the manufacturer's protocol. Briefly, 2 μ g siRNA of each was mixed with 4 μ l enhancer R and 100 μ l buffer EC-R, incubated at RT for 5 min, and then spun down to collect supernatant. Then 8 μ l transMessenger transfection reagent was added to this supernatant. The mixture was vortexed and incubated for 10 min at RT. AMs (3 \times 10⁶ cells) were washed with sterile PBS once, and 900 μ l medium without serum and antibiotics together with siRNA mixture were added into the culture and incubated for 3–4 h at 37°C/5% CO₂. The culture was washed with sterile PBS once and added back complete

medium 1640 with 10% fetal bovine serum (FBS) and 1% PS/AMs and incubated for 60 h at 37°C in 5% CO₂.

PAK1 Protein Phosphorylation Detection

For PAK-1 protein and phosphorylated protein detection, AMs were incubated for 24 h in low FBS (0.4%) complete medium and then incubated with LPS for up to 60 min, or zymosan or *Pneumocystis* for 15 min. Cytoplasmic proteins were isolated and resolved by Western blot using appropriate specific antibodies. Results were compared with unstimulated AMs. *Pneumocystis*-mediated PAK phosphorylation was examined in the presence and absence of a general small GTPase inhibitor (*C. diff* B toxin) or specific Rho-associated protein kinase (ROCK) inhibitor, Y-27632 (Uehata *et al.*, 1997).

Statistical Analysis

Experimental conditions were performed in duplicate or triplicate and repeated with AMs from at least three different individual. Data were analyzed with an Apple G3 Power PC computer utilizing StatView (SAS Institute, Cary, NC) and INSTAT2 (GraphPad Software, San Diego, CA) statistical software. Nonparametric data were analyzed by Fisher Exact Test or ANOVA. Statistical significance was accepted for $p < 0.05$.

RESULTS

Study Subjects

Bronchoscopy was performed on 24 subjects, including 13 healthy individuals (mean age 37 ± 11 y, including 4 females, 5 smokers) and 11 asymptomatic HIV+ subjects (mean age 34 ± 12 y, including 4 female, 9 smokers). None of the individuals had evidence for active respiratory disease. For the HIV+ subjects, peripheral blood CD4 lymphocytes counts were 150-1000 cells/mm³, HIV risk factors included IVDU and homosexual exposures, all were prescribed highly active antiretroviral therapy (HAART), all had undetectable serum viral load (<50 HIV-1 RNA copies/ml), and none experienced a prior opportunistic pneumonia.

AM F-actin Polymerization during Phagocytosis of Unopsonized *Pneumocystis* Organisms

Actin polymerization is a critical component of phagocytosis (Aderem, 2002), but the role in *Pneumocystis* phagocytosis has not been previously demonstrated. To determine whether *Pneumocystis* phagocytosis induces focal F-actin polymerization, AMs from healthy individuals were incubated with unopsonized *Pneumocystis* organisms, and macrophages were stained with rhodamine-phalloidin (which stains polymerized actin). As expected, after incubation with IgG-opsonized *Pneumocystis*, focal F-actin-rich cytoplasmic membrane was evident at points of contact with the opsonized *Pneumocystis* organisms (Figure 1). In comparison, unopsonized *Pneumocystis* also induced reorganization of the actin-based cytoskeleton at points of contact (Figure 1C). No focal F-actin polymerization was evident in response to *Pneumocystis* in the presence of cytochalasin B (an inhibitor of actin polymerization), *C. difficile* toxin (a general inhibitor of RhoGTPases), or Y27632 (specific inhibitor for Rho kinase, ROCK). These data demonstrate that incubation of unopsonized *Pneumocystis* organisms with AMs from healthy individuals induced focal F-actin polymerization, and *Pneumocystis*-induced F-actin assembly was dependent on small RhoGTPases, especially Rho.

Pneumocystis Phagocytosis Activates Cdc42, Rac1, and Rho in AMs from Healthy Individuals

Experiments were next conducted to determine whether *Pneumocystis*-mediated F-actin polymerization was associated with small RhoGTPase activation, with focus on Cdc42, Rac1, and Rho (Caron and Hall, 1998). AMs were incubated with unopsonized *Pneumocystis* organisms up to 60 min, cell lysates prepared, and Western blot determinations were performed to detect both total and GTP-(activated) forms of

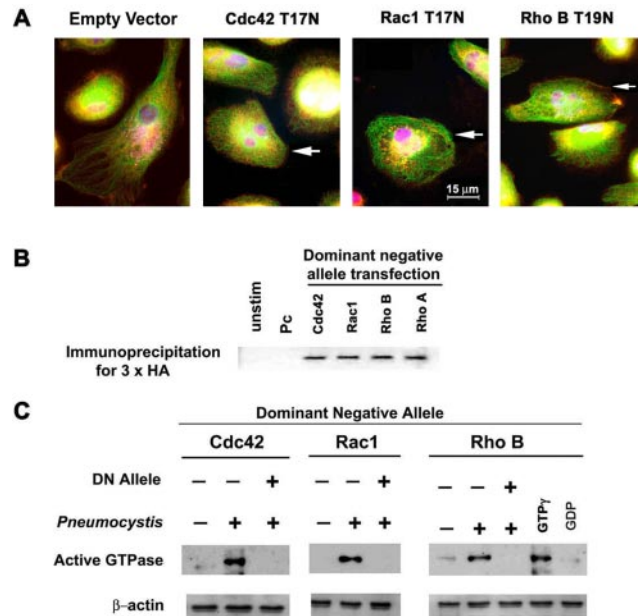


Figure 3. Transfection of AMs from healthy individuals with GTP-binding-deficient alleles of Cdc42 (Cdc42T17N), Rac (Rac1T17N), RhoA (RhoAT19N), or RhoB (RhoBT19N). (A) Immunofluorescence microscopy images illustrate AMs morphology after transfection with empty vector or dominant-negative alleles. Red color indicates 3 \times HA antibody staining of vector used for transfection (arrows indicate transfected cells stained red). Green staining demonstrates intact cellular tubulin lattice structure, and oval blue-magenta DAPI stain indicates macrophage nuclei. (B) Immunoprecipitation of DN proteins in AMs transfected with individual DN alleles detected by anti-3 \times HA antibody. (C) Detection of AM Cdc42, Rac1, and Rho protein activation by Western blot after incubation with unopsonized *Pneumocystis* (15 min at 37°C in 5% CO₂) in the presence and absence of dominant-negative (DN) alleles. The lanes marked GTP γ and GDP served as positive and negative controls for the assay.

Cdc42, Rac1, and Rho proteins. Cdc42, Rac1, and Rho proteins were detected in all cell lysates examined (Figure 2). Unstimulated AMs demonstrated low constitutive activation of Cdc42 and Rho and absent constitutive phosphorylation of Rac1. Incubation with unopsonized *Pneumocystis* resulted in activation of AM Cdc42, Rac1, and Rho. Cdc42 was detected within 1 min of incubation with *Pneumocystis* and peaked by 10 min. Rac1 activation was detected at 3 min and peaked by 15 min. Rho activation demonstrated a bimodal response, with early detection and peak by 1 min, followed by decline, and another peak by 15 min. These data demonstrate that unopsonized *Pneumocystis* organisms induce AM Cdc42, Rac1, and Rho activation in a time-dependent manner, with early activation of Cdc42 and Rho, and relatively delayed activation of Rac1.

Phagocytosis of *Pneumocystis* Organisms Impaired by Dominant Negative Constructs for Cdc42 and RhoB

Next, experiments investigated whether AM phagocytosis of *Pneumocystis* organisms was dependent on functional RhoGTPases.

AMs from healthy individuals were transfected with functional inhibitors of specific RhoGTPases, including GTP-binding-deficient alleles of Cdc42 (Cdc42T17N), Rac1 (Rac1T17N), RhoA (RhoAT19N), or RhoB (RhoBT19N).

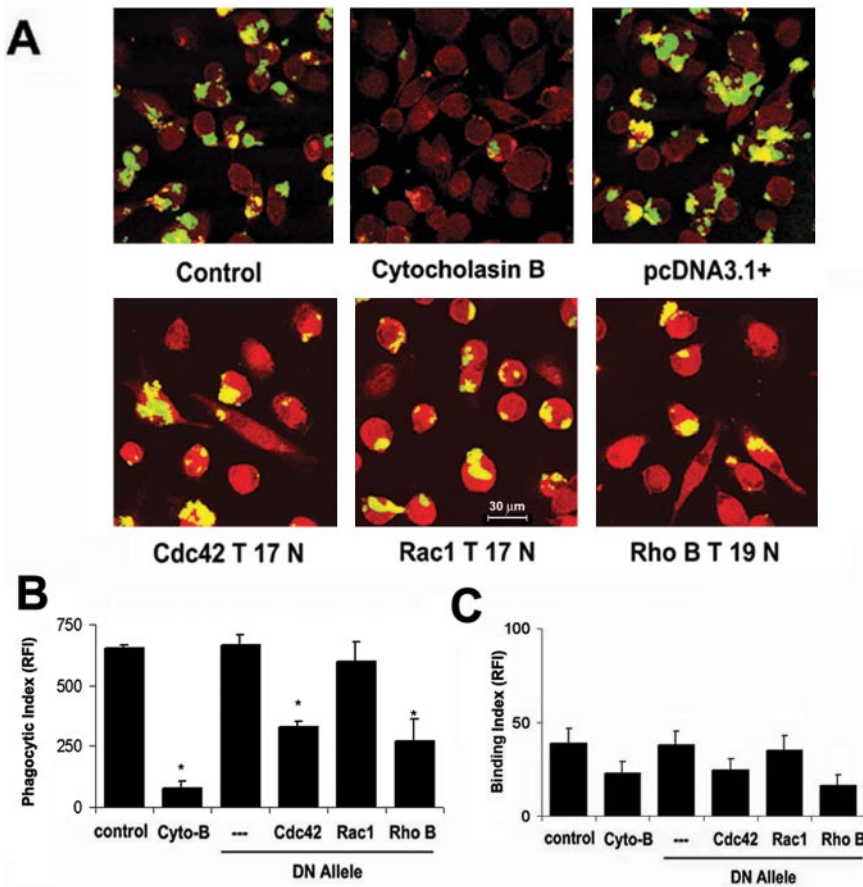


Figure 4. *Pneumocystis* phagocytosis mediated by macrophage Cdc42 and RhoB. AMs were incubated with FITC-labeled *Pneumocystis* organisms for 60 min at 37°C in 5% CO₂, and phagocytosis was determined as described in *Materials and Methods*. (A) Representative confocal microscopy images of adherent AMs from healthy individuals in the absence and presence of cytocholasin B (cyto-B), pcDNA3.1+ (empty vector), or individual DN RhoGTPase constructs. *Pneumocystis* organisms appear yellow-green. Quantitative analysis for *Pneumocystis* phagocytosis (B) and binding (C) by AMs from healthy individuals. Phagocytic Index and Binding Index expressed as relative fluorescence intensity (RFI; values are mean ± SEM; n = 3) *p < 0.05 compared with control.

Transfection of human AMs with these dominant negative constructs did not alter cell morphology (Figure 3A), and individual dominant negative proteins were readily detected by immunoprecipitation in AMs transfected with individual dominant-negative alleles (Figure 3B). Incubation of AMs with unopsonized *Pneumocystis* resulted in activation of Cdc42, Rac1 and Rho (Figure 3C), whereas incubation of dominant-negative transfected AMs with unopsonized *Pneumocystis* did not result in activation of Cdc42, Rac1, or Rho. These data demonstrate successful transfection of human AMs with functional inhibitors of Cdc42, Rac1, and Rho.

Next, to determine the role of specific GTPases in *Pneumocystis* phagocytosis, AMs from healthy individuals transfected with individual dominant negative constructs were incubated with FITC-labeled *Pneumocystis* organisms and phagocytosis was determined by confocal microscopy as previously described (Koziel *et al.*, 1998a). AMs from healthy individuals could bind and phagocytose *Pneumocystis* organisms (Figure 4A), and phagocytosis was markedly reduced in the presence of cytocholasin B (inhibitor of actin polymerization). Transfection of AMs with an empty construct (pcDNA3.1+) did not alter *Pneumocystis* binding and phagocytosis. In comparison, *Pneumocystis* binding and phagocytosis was reduced in the presence of functional inhibitors of Cdc42 and RhoB by qualitative assessment (Figure 4A). Quantitative analysis revealed a significant reduction in *Pneumocystis* phagocytosis in AMs transfected with Cdc42 and RhoB, but not Rac1 (Figure 4B) or RhoA (unpublished data). In contrast, *Pneumocystis* binding was not significantly influenced by the dominant-negative alleles (Figure 4C). These data demonstrate that phagocytosis of

unopsonized *Pneumocystis* organisms by human AMs was dependent on functional Cdc42 and RhoB.

Cdc42 and RhoB Activation Mediated through AM Mannose Receptors

Prior studies demonstrated that transfection of nonphagocytic COS cells with cDNA for human mannose receptor allowed *Pneumocystis* phagocytosis, and mannose receptor inhibition markedly reduced *Pneumocystis* binding and phagocytosis by AMs (Ezekowitz *et al.*, 1991). To determine the contribution of AM mannose receptors to *Pneumocystis* phagocytosis and RhoGTPase activation, mannose receptor functional “knock-down” experiments were performed by sequence-specific, posttranslational gene silencing using short interfering RNA (siRNA) in human AMs.

In general, targeted gene silencing (functional “knock-down”) of the human AM mannose receptors reduced cellular mannose receptor protein (Figure 5A), but did not influence the expression of Cdc42, Rac1, and Rho proteins, and constitutive activation of Cdc42, Rac1, and Rho were low in unstimulated cells (Figure 5). Incubation with unopsonized *Pneumocystis* did not increase AM Cdc42 activation above unstimulated conditions (Figure 5A). As control conditions, incubation with IgG-opsonized *Pneumocystis* (recognized through Fc-γ receptors) increased Cdc42 activation compared with unstimulated cells. In comparison, Rac1 activation was increased in response to both unopsonized *Pneumocystis* and IgG-opsonized *Pneumocystis* (Figure 5B). Similar to the Cdc42 response, Rho activation was not increased in response to unopsonized *Pneumocystis*, but was increased in response to IgG-opsonized *Pneumocystis* (Figure

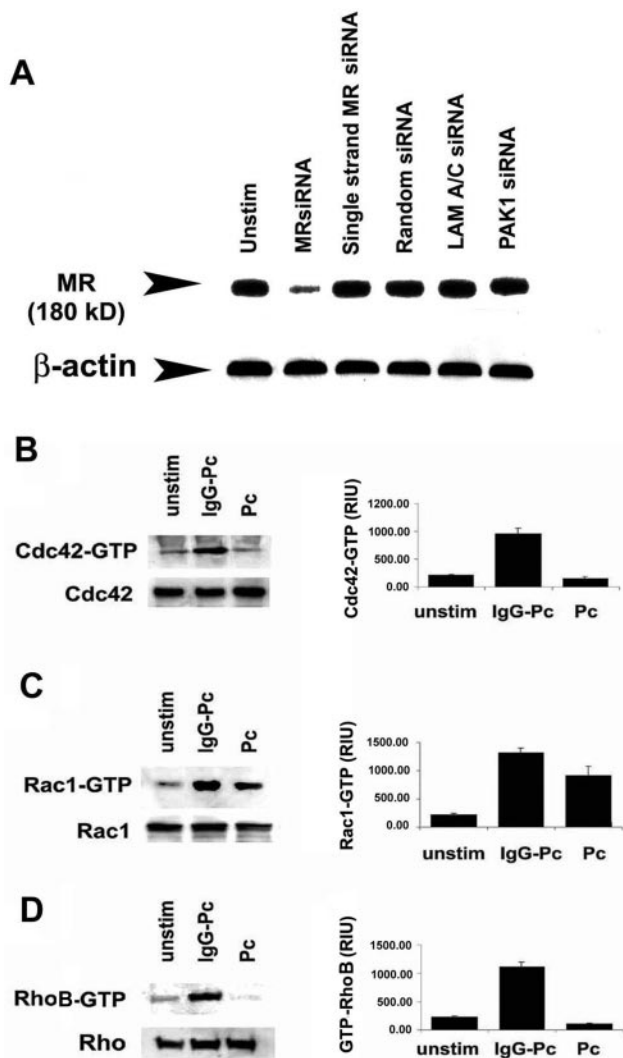


Figure 5. Cdc42 and RhoB activation mediated through AM mannose receptors. Targeted AM mannose receptor gene silencing by siRNA followed by incubation with unopsonized *Pneumocystis* for 15 min at 37°C in 5% CO₂. (A) Reduced mannose receptor total cellular protein in AMs after siRNA gene silencing. Mannose receptor protein was not influenced by single-stranded MR siRNA, random siRNA, or LAM A/C siRNA (served as negative controls). PAK1 siRNA represents gene silencing of AM PAK1 (which did not reduce mannose receptor and demonstrates specificity). (B–D) Influence of *Pneumocystis* on AM Rho GTPase activation after mannose receptor gene silencing. Western blot of cell lysates performed to detect both total and activated (–GTP) forms of (B) Cdc42, (C) Rac1, and (D) RhoB. Western blots are shown for AMs from one healthy individual and are representative of experiments from at least three different individuals. Corresponding bar graphs represent densitometry quantitative analysis for Cdc42-GTP, Rac1-GTP, and RhoB-GTP (mean ± SEM; n = 3) expressed as relative intensity units (RIU).

5C). These data demonstrated that human AM Cdc42 and RhoB activation in response to unopsonized *Pneumocystis* organisms is mediated predominately through mannose receptors.

HIV-1 Infection Impairs Focal F-actin Polymerization in Response to Unopsonized *Pneumocystis* Organisms

To consider the clinical relevance of these findings, experiments next examined the role of F-actin polymerization in

response to nonopsonized *Pneumocystis* using AMs from HIV+ persons (recognized clinical risk factor for *Pneumocystis* pneumonia). Compared with AMs from healthy individuals, incubation of unopsonized *Pneumocystis* organisms with AMs from asymptomatic HIV+ persons did not induce focal F-actin polymerization at points of contact (Figure 6). Similarly, unopsonized *Pneumocystis* organisms did not induce F-actin polymerization in AMs from healthy individuals after in vitro HIV infection. These data demonstrate that *Pneumocystis*-induced F-actin assembly is impaired in AMs from HIV+ persons, and HIV infection is sufficient to impair *Pneumocystis*-mediated F-actin polymerization in AMs.

HIV Infection Impairs Cdc42 and Rho Activation in AMs in Response to *Pneumocystis* Organisms

To test the hypothesis that altered mannose receptor expression may influence *Pneumocystis*-mediated small GTPase activation, experiments next examined Cdc42, Rac1, and Rho activation in AMs from HIV+ subjects, and in AMs from healthy individuals after in vitro HIV infection (conditions associated with reduced mannose receptor expression and function).

Cdc42 protein was detected in the cytoplasm of intact cells and cytosolic extracts from all three groups of AMs studied (Figure 7). For AMs from healthy individuals, Cdc42 activation was increased in response to unopsonized *Pneumocystis* organisms, in addition to zymosan (alternative ligand for mannose receptors) and PMA (mannose receptor-independent ligand). In comparison, AMs from HIV+ persons demonstrated reduced Cdc42 activation in response to *Pneumocystis* organisms (and also reduced in response to zymosan and PMA). After in vitro HIV infection, Cdc42 activation was completely inhibited in response to *Pneumocystis* organisms and zymosan and markedly reduced in response to PMA.

In comparison, Rac1 activation was not significantly impaired in response to *Pneumocystis*, zymosan, or PMA in AMs from HIV+ persons, although Rac1 activation was completely impaired in response to *Pneumocystis* and zymosan (but not PMA) after in vitro HIV infection. In contrast, Rho activation was not increased in response to *Pneumocystis* organisms (or zymosan and PMA) in AMs from HIV+ persons or after in vitro HIV infection. These data demonstrated that Cdc42 and Rho activation was reduced in AMs from HIV+ persons in response to unopsonized *Pneumocystis* organisms, whereas Rac1 activation was not significantly reduced. Furthermore, in vitro HIV infection of AMs (a condition associated with marked reduction in mannose receptor expression and function; Koziel *et al.*, 1998a) was sufficient to impair *Pneumocystis*-mediated Cdc42, Rac1, and Rho activation.

HIV Infection Impairs p21-activated Kinase Phosphorylation in Response to *Pneumocystis* Organisms

The p21-activated kinases (PAK) are important serine/threonine signaling molecules regulating a variety of cellular function (including cytoskeletal dynamics), and PAK are stimulated by activated Cdc42 and Rac (Bokoch, 2003). To determine the downstream effects of *Pneumocystis*-mediated Cdc42, Rac1, and Rho activation, experiments next measured PAK phosphorylation in response to unopsonized *Pneumocystis*. Incubation of AMs from healthy individuals with unopsonized *Pneumocystis* organisms increased PAK-1 phosphorylation (activated PAK) in a time-dependent manner, detected at 10 min and peaked at 15 min (Figure 8A). Pretreatment of the AMs with *C. difficile* toxin B (general inhibitor of Rho family GTPases) or Y-27632 (specific inhib-

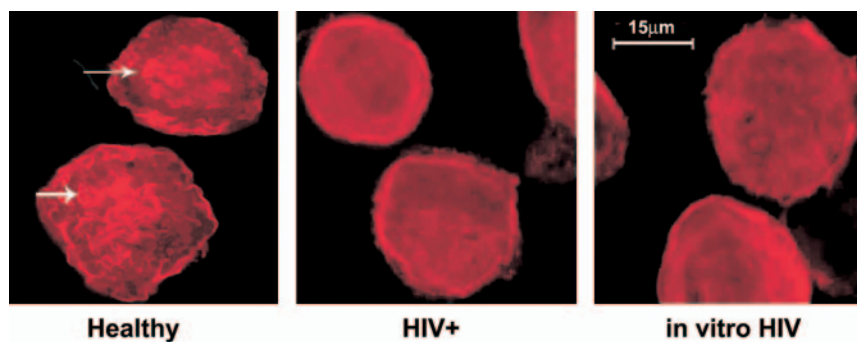


Figure 6. HIV infection impairs AM F-actin polymerization in response to unopsonized *Pneumocystis* organisms. Immunofluorescence microscopy images of isolated AMs after incubation with *Pneumocystis* organisms (ratio 10:1 organisms to macrophages) for 20 min at 37°C in 5% CO₂. Polymerized F-actin was detected by rhodamine-phalloidin (red color) as described in *Materials and Methods*. Focal F-actin polymerization was evident in AMs at points of contact with organisms (arrows) in healthy individuals, but not in AMs from HIV+ subjects (HIV+) or AMs from healthy individuals after in vitro HIV infection (in vitro HIV). Representative images of at least three individuals from each group.

itor for Rho kinase, ROCK) markedly reduced PAK phosphorylation in response to *Pneumocystis* (Figure 8B). These data demonstrated that unopsonized *Pneumocystis* organisms promote AM PAK-1 phosphorylation, and *Pneumocystis*-mediated PAK-1 phosphorylation was dependent on Rho GTPases, especially Rho.

In marked contrast to AMs from healthy individuals, PAK-1 phosphorylation was markedly reduced or absent in AMs from asymptomatic HIV+ persons or in AMs after in vitro HIV infection (Figure 8C). PAK-1 protein levels were also markedly reduced in AMs from HIV+ subjects and healthy individuals after in vitro HIV infection. Taken together, these data demonstrate that impaired *Pneumocystis*-mediated Cdc42 and Rho phosphorylation in AMs from HIV+ persons was associated with reduced PAK phosphorylation (an effector molecule for Cdc42), and in vitro HIV infection was sufficient to reduce *Pneumocystis*-mediated PAK phosphorylation.

Role of RhoGTPase Effector Molecules, PAK1, and ROCK on *Pneumocystis* Phagocytosis by Human AMs

To further define the role of the Cdc42 effector, PAK1, and the Rho effector, ROCK, on *Pneumocystis* phagocytosis, experiments used siRNA functional gene silencing of AM PAK1 and mannose receptors, and the specific Rho kinase inhibitor Y27632. Functional gene silencing of AM PAK1 reduced PAK cellular protein (Figure 9A), but only partially reduced *Pneumocystis* phagocytosis (Figure 9B). Functional gene silencing of mannose receptors or inhibition of the Rho kinase ROCK significantly reduced *Pneumocystis* phagocytosis (Figure 9B). *Pneumocystis* binding was not influenced by gene silencing of PAK or mannose receptors, but was significantly reduced in the presence of the ROCK inhibitor, Y27632 (Figure 9C). These data suggest that *Pneumocystis* phagocytosis is only partially dependent on PAK1 activation, but is dependent on the Rho effector molecule, ROCK.

DISCUSSION

These data demonstrate that in AMs from healthy individuals, phagocytosis of unopsonized *Pneumocystis* was associated with focal F-actin polymerization and small Rho GTPase activation. Phagocytosis of unopsonized *Pneumocystis* was mediated predominantly through mannose receptors (as determined by targeted mannose receptor siRNA gene silencing) and was critically dependent on Cdc42 and RhoB activation. Unopsonized *Pneumocystis* also promoted PAK-1 phosphorylation, an important downstream effector molecule, and PAK phosphorylation was dependent in part on RhoGTPase activation, especially Rho. In contrast, HIV infection of AMs (a condition associated with reduced man-

nose receptor expression and function) was associated with impaired focal F-actin polymerization, reduced Cdc42 and Rho activation, and markedly reduced PAK-1 phosphorylation in response to unopsonized *Pneumocystis* organisms. These data provide a molecular mechanism for mannose receptor-mediated phagocytosis of unopsonized *Pneumocystis* organisms by human AMs. Furthermore, impaired AM Cdc42 and Rho activation may provide the molecular basis for impaired *Pneumocystis* phagocytosis in the context of HIV infection (Koziel *et al.*, 1998a), which may contribute to the pathogenesis of opportunistic pneumonia.

The current study represents the first to examine the molecular mechanism of human AM phagocytosis of unopsonized *Pneumocystis* organisms. Phagocytosis of *Pneumocystis* requires focal F-actin polymerization, Cdc42 and Rho activation, promotes PAK1 activation and requires the Rho effector molecule ROCK. A recent study in a murine model identified the dectin-1 receptor for β -glucan receptor as the critical receptor mediating *Pneumocystis* phagocytosis and killing by macrophages (Steele *et al.*, 2003), although the role of RhoGTPases was not investigated. Although macrophage β -glucan receptors may represent an important component of an effective host response to *Pneumocystis* in the rodent model, for human AM *Pneumocystis* recognition is mediated predominantly via mannose receptors. However, defining the specific role of human AM dectin-1 receptor in phagocytosis and signaling requires further investigation.

The current study suggests that the molecular mechanism for opsonin-independent macrophage phagocytosis may be distinct from opsonin-dependent phagocytosis. Fc γ R-mediated phagocytosis of appropriately opsonized particles may be mediated predominantly by Cdc42 and Rac1, whereas C3R-mediated phagocytosis of appropriately opsonized particles may be mediated primarily by Rho (Caron and Hall, 1998). The distinction, however, may not be absolute, as Rho may also contribute to Fc γ R-mediated phagocytosis (Hackam *et al.*, 1997). Other investigations of opsonin-independent phagocytosis demonstrated that phagocytosis of unopsonized *Borrelia burgdorferi* by human macrophages was mediated predominantly by Cdc42 and in part by Rac1, but not Rho (Linder *et al.*, 2001). Macrophage phagocytosis of unopsonized *Pseudomonas aeruginosa* was mediated by Cdc42 and Rac (although Rho was not specifically investigated; Lee *et al.*, 2000). In the current study, *Pneumocystis* phagocytosis was mediated via Cdc42 and Rho, but not Rac1. Taken together, although Cdc42 may be an important component to opsonin-independent phagocytosis, the role of other specific RhoGTPases in opsonin-independent phagocytosis of pathogens may depend on multiple factors, including the nature of the pathogen, nature and species origin of the cells, and specific phagocytic receptor(s) en-

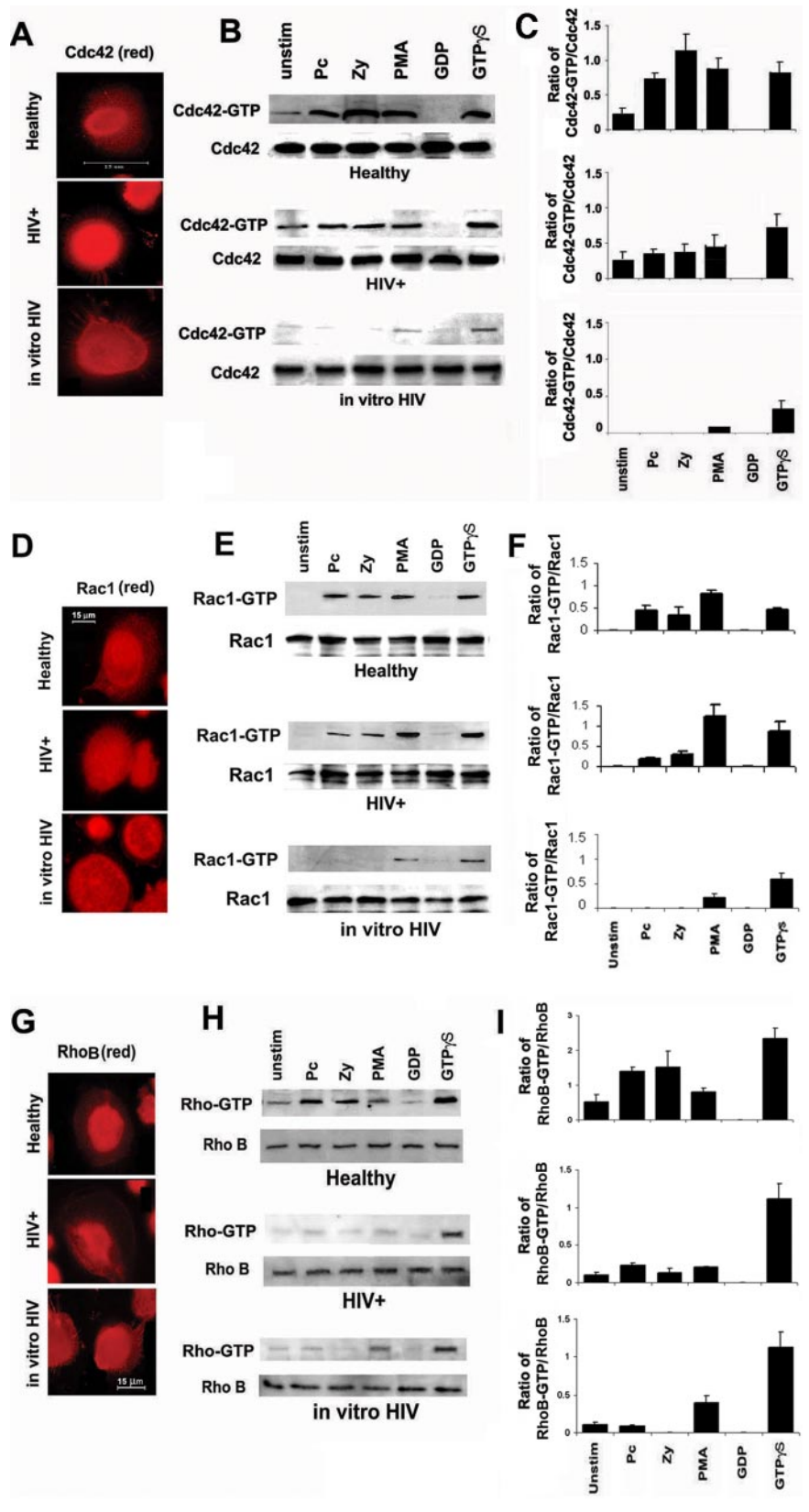


Figure 7. HIV infection impairs AM Cdc42 and Rho activation in response to unopsonized *Pneumocystis* organisms. AMs were incubated with unopsonized *Pneumocystis* organisms (Pc), zymosan (Zy), or PMA for 15 min at 37°C in 5% CO₂. AMs were from healthy individuals, asymptomatic HIV-infected persons (HIV+), or healthy individuals after in vitro HIV infection (in vitro HIV). Immunofluorescence microscopy images demonstrated similar cellular localization for (A) Cdc42, (D) Rac1, and (G) Rho with representative cells for healthy, HIV+, and in vitro HIV. Western blot of cell lysates was performed to detect both total and activated (-GTP) forms of Cdc42 (B), Rac1 (E), and Rho (H) for healthy, HIV+, and in vitro HIV. The corresponding bar graphs represent densitometry quantitative analysis for Cdc42-GTP (C), Rac1-GTP (F), and Rho-GTP (I) for each group of healthy, HIV+, and in vitro HIV. Data are normalized as a ratio of the active (-GTP) RhoGTPase over the total RhoGTPase and are expressed as relative units (mean ± SEM). Data are representative of experiments from three different individuals in each group (n = 3).

gaged. Further studies are required to completely define the role of specific RhoGTPases in opsonin-independent phagocytosis.

The current study represents the first to demonstrate phagocytic signal transduction pathways mediated by mannose receptors. Mannose receptors belong to a family of type

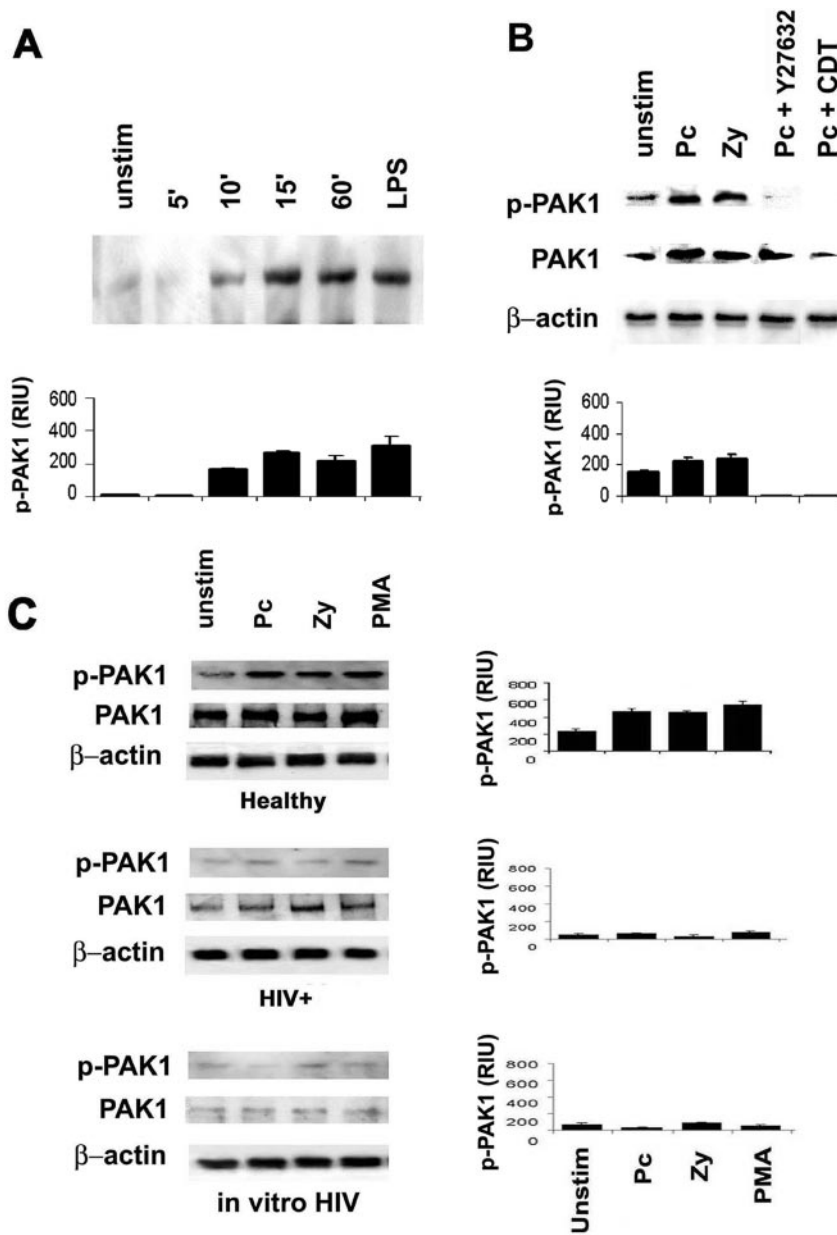


Figure 8. HIV infection impairs PAK-1 activation in response to *Pneumocystis* organisms. (A) AMs from healthy individuals were incubated with unopsonized *Pneumocystis* (5:1) up to 60 min at 37°C in 5% CO₂, and cell lysates were examined for phosphorylated PAK1 (p-PAK1) by Western blot. LPS served as a positive control for PAK1 phosphorylation. (B) AMs from healthy individuals were incubated with unopsonized *Pneumocystis* (5:1) or zymosan (5:1) in the presence of absence of Y26371 (the specific inhibitor of Rho kinase, Rock) or *C. diff* toxin B (CDT; general RhoGTPase inhibitor) for 15 min at 37°C in 5% CO₂, and cell lysates were examined for total (PAK1) and phosphorylated PAK1 (p-PAK1) by Western blot using specific antibody. (C) AMs were incubated with *Pneumocystis* (Pc), zymosan (Zy), or PMA for 15 min at 37°C in 5% CO₂, and Western blots on cell lysates were performed to detect both total (PAK1) and phosphorylated PAK1 (p-PAK1) in AMs from healthy, HIV+, and healthy after in vitro HIV. For all Western blots, the corresponding bar graphs represent densitometry quantitative analysis for p-PAK1 for each group of healthy, HIV+, and in vitro HIV expressed as relative intensity units (RIU; mean ± SEM). The depicted Western blots represent cells from one healthy individual and are representative of at least three different subjects examined.

I transmembrane C-type lectin receptors that mediate both endocytosis and phagocytosis (Ezekowitz, 1992). Mannose receptors contain multiple C-type lectin-like domains each capable of binding specific monosaccharides such as mannose, fucose, and *N*-acetylglucosamine. Although endocytosis requires signaling through a conserved tyrosine-based FENTLY motif in the cytoplasmic tail of the receptor (East and Isacke, 2002), the signaling molecules mediating phagocytosis and the downstream cell signaling pathways remain undefined (Fraser *et al.*, 1998). The current study demonstrates that mannose receptor-mediated phagocytosis was associated with focal F-actin polymerization, dependent on Cdc42 and Rho activation, and dependent on the Rho effector molecule, Rock. Rac1 activation was not critical for *Pneumocystis* phagocytosis (as introduction of nonfunctional Rac1 dominant-negative allele did not influence *Pneumocystis* phagocytosis). The relative importance of mannose receptors in mediating *Pneumocystis* phagocytosis by human AMs

was supported by targeted siRNA gene silencing experiments and the observations in AMs from HIV-infected persons (a disease state characterized by reduced macrophage mannose receptor surface expression; Kozziel *et al.*, 1998a).

Findings in the current study suggests that the initial interaction of unopsonized *Pneumocystis* organisms with AMs promotes rapid Cdc42 activation, in addition to Rho activation (early portion of biphasic response), followed by a relatively delayed Rac1 activation, and further Rho activation (delayed portion of biphasic response). The data suggest the early activation of Cdc42 and Rho are mediated predominantly through mannose receptors (as supported by the siRNA gene silencing experiments) and that the delayed responses of Rac and Rho were mannose receptor independent (and perhaps mediated by other macrophages receptors such as dectin-1; Brown and Gordon, 2001; Steele *et al.*, 2003), although additional experiments are required to further define these specific events.

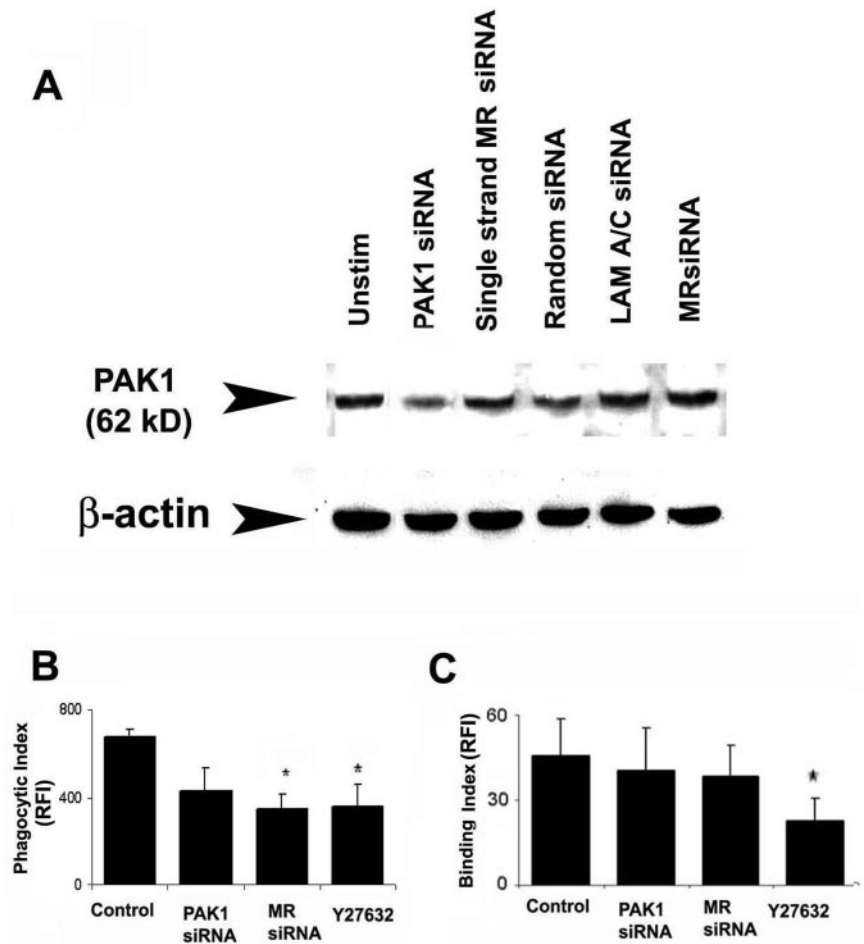


Figure 9. The role of RhoGTPase effector molecules, PAK1 and ROCK, on *Pneumocystis* phagocytosis by human AMs. (A) Western blot analysis of cellular PAK1 protein in human AMs after siRNA gene silencing. PAK1 protein was not influenced by single-stranded PAK siRNA, random siRNA, or LAM A/C siRNA (served as negative controls). MR siRNA represents gene silencing of AM mannose receptor (which did not reduce PAK1 and demonstrates specificity). *Pneumocystis* phagocytic index (B) and binding index (C) of AMs after functional siRNA gene silencing of PAK1 (PAK1 siRNA), mannose receptor (MR siRNA), or specific pharmacological inhibition of the Rho kinase ROCK (by Y27632). Phagocytic Index and Binding Index expressed as relative fluorescence intensity (RFI; values are mean \pm SEM; n = 3) *p < 0.05 compared with control.

The mechanism for impaired RhoGTPase activation in HIV infection was not completely defined. HIV infection was sufficient to impair RhoGTPase activation in response to *Pneumocystis*. Impaired RhoGTPase activation may in part be attributed to reduced mannose receptor surface expression, based on reduced mRNA (Kozziel *et al.*, 1998a) or through increased release of the mannose receptor ectodomain (Fraser *et al.*, 2000). Alternatively, impaired RhoGTPase activation may be due to direct influence by HIV gene products that can influence cellular receptor expression and signal transduction pathways (Emerman and Malim, 1998). HIV-1 *tat* may down-regulate mannose receptor transcription (Caldwell *et al.*, 2000). HIV-1 *vpu* impedes NF- κ B activation by inhibiting I- κ B degradation (Bour *et al.*, 2001) in T-cell lines. HIV-1 *nef* may alter ASK1 signal transduction pathways (Geleziunas *et al.*, 2001) and down-regulate NF- κ B signaling in T-cell lines (Bandres and Ratner, 1994), reduce mannose receptor surface expression on dendritic cells (Quaranta *et al.*, 2002), and down-modulate Fc γ -R expression on monocyte-derived macrophages (De *et al.*, 1998). The influence of specific HIV-1 gene products on AM RhoGTPase signal transduction pathways has not been previously investigated. The observed alteration in Cdc42, Rac1, and Rho in the current study may thus reflect the influence of one or more HIV-related factors and represents an area of active investigation.

The role of other receptors implicated in the AM recognition of *Pneumocystis*, including fibronectin (Pottratz and

Martin, 1990) and immunoglobulin receptors (Masur and Jones, 1978; von Behren and Pesanti, 1978) were not specifically investigated. The observed Cdc42 and Rac phosphorylation in response to IgG-opsonized *Pneumocystis* in AMs with targeted gene silencing of the mannose receptor suggests that the Fc γ R pathway was intact. Other limitations of these studies include the possibility that the observed response of human AMs to rodent-derived *Pneumocystis* may reflect in part the origin of the organisms. Unfortunately, in the absence of reliable culturing methods, *Pneumocystis* of human origin are not available (Sloand *et al.*, 1993). However, the recent observation that rat-derived *Pneumocystis* elicit similar chemokine and NF- κ B responses comparing human AMs to rat AMs in part validates the use of this model (Zhang *et al.*, 2004). Contaminating rat-derived adherent proteins may be in part responsible for the observed RhoGTPase activity, although the *Pneumocystis* organisms used in these studies are relatively free of host proteins such as surfactant protein-A (Kozziel *et al.*, 1998b). Because the *Pneumocystis* preparation is composed primarily of the trophic forms, the current findings may represent the predominant macrophage response to the *Pneumocystis* trophic form and not the cyst or other form. Finally, the possibility that in vitro observations may not accurately reflect events in vivo should be considered.

In conclusion, these data demonstrate that in AMs from healthy individuals, phagocytosis of unopsonized *Pneumocystis* was mediated predominantly through mannose receptors and was critically dependent on Cdc42 and especially

RhoB activation. These data provide a molecular mechanism for mannose receptor-mediated phagocytosis of unopsonized *Pneumocystis* organisms, and the mechanism is distinct from opsonin-dependent phagocytic receptors. Furthermore, impaired AM Cdc42 and Rho activation may provide the molecular basis for impaired *Pneumocystis* phagocytosis in AMs from HIV+ persons (Kozziel *et al.*, 1998a). Recognizing the spectrum of pathogens recognized by mannose receptors (including *Mycobacterium tuberculosis*, *Cryptococcus neoformans*, and *Pneumocystis*), impaired AM mannose receptor-mediated phagocytosis in the context of HIV infection may contribute to the pathogenesis of opportunistic pneumonia.

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