Lipid Rafts Regulate Lipopolysaccharide-induced Activation of \( \text{Cdc42} \) and Inflammatory Functions of the Human Neutrophil*

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Lipid rafts are cholesterol-rich membrane microdomains that are thought to act as coordinated signaling platforms by regulating dynamic, agonist-induced translocation of signaling proteins. They have been described to play a role in multiple prototypical cascades, among them the lipopolysaccharide pathway, and to host multiple signaling proteins, including kinases and low molecular weight G-proteins. Here we report lipopolysaccharide-induced activation of the Rho family GTPase Cdc42, and we show its activation in the human neutrophil to be mediated by a p38 mitogen-activated protein kinase-dependent mechanism. Subcellular fractionation reveals that lipopolysaccharide induces translocation of Cdc42 to lipid rafts, where it and p38 are both found to be activated. By contrast, lipopolysaccharide causes translocation of Rac from the polymorphonuclear leukocyte (PMN) rafts and does not induce its activation. With the use of methyl-\( \beta \)-cyclodextrin, a cholesterol-depleting agent that reversibly disrupts rafts, we confirm an important regulatory role for rafts in the activation state of p38 and Cdc42 and in the Rho GTPase-dependent functions superoxide anion production and actin polymerization. Methyl-\( \beta \)-cyclodextrin induces activation of p38 and Cdc42, but not Rac, in the nonstimulated PMN, yet inhibits subsequent lipopolysaccharide-induced activation of p38 and Cdc42. In parallel, methyl-\( \beta \)-cyclodextrin primes the human PMN for subsequent superoxide release triggered by the formylated bacterial tripeptide formyl-Met-Leu-Phe, and induces actin polymerization in a subcellular distribution distinct from that induced by lipopolysaccharide. In sum, these findings provide evidence for an important regulatory role of cholesterol in both transmission of the lipopolysaccharide signal and the inflammatory phenotype of the human neutrophil.

Lipid rafts are cholesterol- and glycosphingolipid-rich membrane microdomains that are thought to function as coordinated signaling platforms in cells through their regulation of dynamic, agonist-induced translocation of specific signaling proteins (1, 2). A variety of signaling components, including receptors (e.g. epidermal growth factor, platelet-derived growth factor, and endothelin receptors), kinases (e.g. Lyn, Syk, and protein kinase C), phosphatases (e.g. Syp), and both heterotrimeric and low molecular weight G-proteins (e.g. Rac, Cdc42, and Rho) have been localized to rafts (1, 2). Raft compartmentalization is thought to play an important role in regulating the activation state of, and dynamic interactions among, proteins. A centrally important implication of localization of signaling events to lipid rafts is that such molecular interactions, like the physicochemical organization of rafts themselves, are sensitive to alteration of cellular cholesterol content. By exploiting this property of rafts, multiple investigators (3–5) have used cyclodextrins (cyclic oligomers of glucose that can be used both to extract cholesterol from intact, living cells, as well as to deliver cholesterol to them) as a tool to perturb rafts physically and functionally by cholesterol depletion, and thereby to uncover their native regulatory influence upon cellular signaling phenomena. The mechanism of cyclodextrin-induced effects upon the cell can be confirmed as cholesterol depletion either by co-incubating cells with cyclodextrin and cholesterol (thereby preventing depletion) or by using the cyclodextrin-cholesterol complex as a vehicle to sequentially cholesterol-replete cells that have been depleted by cyclodextrin. Repletion is thought to restore raft structure and function (5).

Although the best established role for rafts as signaling platforms may have been demonstrated in T and B cell receptor activation (6, 7), recent reports indicate lipopolysaccharide (LPS) induced interactions between Toll-like Receptor 4 (TLR4) and other proteins in monocyte rafts (8, 9). This discovery (made possible by the ability to isolate lipid rafts by exploiting their properties of Triton insolubility and relative buoyant density) has ignited interest in using rafts as a window to discover novel signaling interactions in LPS signaling (8, 10). In this light, Rho family GTPases (i.e. Cdc42, Rac, and Rho) have recently been independently reported to be localized to rafts in ECV304 cells (11), to undergo PDGF-induced translocation into fibroblast rafts (Rac and Rho) (12), and to play a role in LPS signaling in an endothelial cell line (13) and in fibroblasts (14). By analogy, Rac1 and Cdc42 have also been re-

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§ The abbreviations used are: LPS, lipopolysaccharide; fMLP, formyl-methionyl-leucyl-phenylalanine; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; mCD, methyl-\( \beta \)-cyclodextrin; PBD, p21 binding domain; PMN, polymorphonuclear leukocyte; TLR, Toll-like Receptor; GTP\( \gamma \)S, guanosine 5’-3’-O-(thio)triphosphate; MES, 2-N-morpholinoethanesulfonic acid; PDGF, platelet-derived growth factor; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); AEBSF, 4-[(2-aminoethyl)benzenesulfonyl]fluoride; PBS, phosphate-buffered saline.
ported to be activated and dynamically recruited to TLR2 in THP-1 cells stimulated with *Staphylococcus aureus* (15). To our knowledge there have been no reports describing LPS-induced activation of Rho GTPases in a human primary immune cell nor in confirming a clear role for lipid rafts in the regulation of these proteins.

The Rho GTPases Rac and Cdc42 have been shown in the neutrophil (PMN) to regulate innate immune response functions, including superoxide anion (O2•−) generation, actin polymerization, and chemotaxis (16, 17). Therefore, we sought in the present study to investigate whether LPS activates Rac and/or Cdc42 in the human PMN and, in parallel, to discern whether rafts exert any regulatory influence upon their activation or upon Rho GTPase-dependent functions. Here we report that LPS activates Cdc42 via a p38 mitogen-activated protein kinase (MAPK)-dependent mechanism and induces recruitment of Cdc42 into rafts, where it and p38 are both found to be activated. Furthermore, we demonstrate a clear role for lipid rafts in regulation of the activation state of p38 and Cdc42 and in regulation of Rho GTPase-dependent functions, specifically O2•− generation and actin polymerization. These findings shed new light upon the basic mechanisms underlying the innate immune response, and raise new and interesting questions about the role of cholesterol in regulating the inflammatory phenotype of the human PMN.

**EXPERIMENTAL PROCEDURES**

*Reagents—* Endotoxin-free reagents and plastics were used in all experiments. PMNs were isolated from whole blood by discontinuous plasma Percoll centrifugation (18) and were resuspended in RPMI 1640 culture medium (BioWhittaker, Walkersville, MD) supplemented with 10 mM Hepes (pH 7.6) and 1% human heat-inactivated platelet-poor plasma. Aprotinin, leupeptin, AEBSF, sodium fluoride, sodium orthovanadate, formyl-methionyl-leucyl-phenylalanine (fMLP), Triton-X-100, sucrose, cytochrome c, MES, 2-amino-2-methyl-1-propanol, parafomaldehyde, and protein A-Sepharose were purchased from Sigma. Methyl-β-cyclodextrin (mCD), cholesterol, and pregnenolone were purchased from Aldrich. p-Nitrophenyl phosphate was purchased from Pierce. SB203580, a p38 inhibitor, was purchased from Calbiochem-Novabiochem. p21 binding domain (PBD)-glutathione S-transferase (GST)-agarose beads, GTP-S, GDP, and 5× MLB lysis/wash buffer were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). NBD-phallacidin and rhodamine-phalloidin were purchased from Molecular Probes (Eugene, OR). *Escherichia coli* oli11111B4 LPS was purchased from List Biological Laboratories, Inc. (product 201, Campbell, CA). The *Limonials* amebocyte lysate (MLL) was purchased from Reliable Diagnostics (Bellmore, NY). Antibodies—Mouse anti-CD14 antibody was purchased from Coulter Corp. (Hialeah, FL). Rabbit anti-phospho-p38 antibody specific for the activation residues of p38 (Thr-180/Tyr-182) was purchased for the activation residues of p38 (Thr-180/Tyr-182) was purchased from Santa Cruz Biotechnology, Inc.) supplemented with 1 μM AEBSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM NaF, and 1 mM Na3VO4, and clarified by centrifugation, where the supernatant was then incubated with EGTA and acetonitrile. The vials were then capped and heated at 60 °C for 45 min. Analysis of the samples was performed by a Finnigan Trace gas chromatography/mass spectrometry system (Thermo Finnigan, Thousand Oaks, CA), using a 15-m ZB-1 column from Phenomenex (Torrance, CA), by injecting 1% of the extract. The mass spectrometer was operated in the electron impact mode. Data were acquired on monitoring, where m/z 388 was monitored for cholesterol and m/z 374 was monitored for the trimethylsilyl ether derivative of 5α-cholesterol. Peak areas of each analyte were measured, and the ratio of the area of the cholesterol-derived ion to that from the internal standard was

**Lipid Raft Isolation—**PMNs (400 × 10⁶) were resuspended at a concentration of 20 × 10⁷/ml in RPMI 1640 supplemented with 10 mM Hepes (pH 7.6) and 1% human heat-inactivated platelet-poor plasma, and either left untreated or stimulated as described. Following stimulation, rafts were isolated by using a modification of the method of Cheng et al. (20). Cells were centrifuged (15,000 rpm, 20 min) and resuspended in 1 ml of ice-cold lysis buffer (25 mM MES (pH 6.5), 150 mM NaCl, 1% Triton X-100, 1 mM AEBSF, 1 mM NaF, 5 μg/ml aprotinin, 1 mM Na3VO4, 2 μg/ml aprotinin, 1 mM Na3VO4, and 5 μg/ml aprotinin. PMNs were then sonicated for 60 s on ice. High density insoluble debris was removed by centrifugation (1000 × g, 10 min, 4 °C). The supernatant (950 μl) was added to 2 ml of 80% sucrose in MBS (25 mM MES (pH 6.5), 150 mM NaCl), gently mixed by vortexing, and then centrifuged through a 10–40% continuous sucrose gradient using an SW41 rotor (20 h, 37,000 rpm, 4 °C). Following centrifugation, 10-ml fractions were harvested from the bottom by puncturing the tube with an 18-gauge needle. Protein concentrations in each fraction were determined by the Bradford Protein Assay (21), and 10 μg of protein from each fraction was electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies for proteins of interest. Raft-containing fractions were identified by fluorescence phosphorescence measurement (22), as well as by immunoblotting for the raft-specific protein flotillin-1 (22, 23).

**Alkaline Phosphatase Assay—**Sucrose density gradient fractions were measured for enzymatic activity of alkaline phosphatase (a raft-specific, glycosylphosphatidylinositol-linked membrane protein (24, 25)) to identify rafts (26). Briefly, 20 μl of each fraction was added to separate wells of a 96-well flat-bottom plate and then mixed with 200 μl of reaction buffer (5 mM p-nitrophenyl phosphate in 100 mM 2-amino-2-methyl-1-propanol, pH 10.0). Reaction mixtures were incubated (15 min, 37 °C), and the absorbance was then read in a microplate reader at 405 nm.

**Cellular Cholesterol Depletion and Repletion—**PMNs were cholesterol-depleted with mCD using a modification of Illangumaran and Hoessli (27). Briefly, cells were resuspended at 5 × 10⁶/ml in Krebs-Ringer phosphate buffer supplemented with 0.2% dextrose and 0.25% delipidated bovine serum albumin, incubated with 5 mM mCD (15 min, 37 °C), washed, and then resuspended. In order to demonstrate the raft specificity of mCD, cholesterol repletion of depleted cells (hereafter, “sequential repletion”) was performed by incubation with mCD for 100 μM cholesterol complex (see below) (15 min, 37 °C), followed by washing and reassociation. As a control for cholesterol repletion, depleted cells were incubated with mCD for 100 μM pregnenolone, a complex ineffective in cholesterol delivery. As an alternative control to sequential repletion, cholesterol depletion and repletion, untreated cells were treated with a concentration of cholesterol (10 μM) complexed to mCD that was confirmed to maintain cellular cholesterol levels constant (hereafter, “iso-repletion,” see under “Results”). 10 mM stock solutions of mCD-cholesterol and mCD-pregnenolone complexes were formed by dissolving 30 mg of cholesterol or 24.7 mg of pregnenolone, respectively, in 400 μl of 2:1 methanol/chloroform, and then dissolving these dropwise in 11 ml of 69.4 mM mCD in PBS preheated to 80 °C. This solution was freeze-dried overnight, and working stock solutions were made in water.

**Cellular Cholesterol and Phospholipid Quantitation—**A mass spectrometric method was developed for cellular cholesterol quantitation. To determine cholesterol content of PMNs, 50 μg/ml mCD (1 ml). An aliquot of this sample (10%) was taken for analysis, and stable isotope-labeled d5-cholesterol (50 ng, Cambridge Isotope Laboratories, Inc., Andover, MA) was then added to this aliquot. After mixing, samples were extracted with 1 ml of iso-octane. A portion of the extract of a stream of N2, and trimethylsilyl derivatives were prepared by heating at 60 °C. Following extraction, the cholesterol-derived ion to that from the internal standard was

| Protein | Relative Cholesterol | Relative Phospholipid |
|---------|---------------------|----------------------|
| Cholesterol 
| Phospholipid 
| Lipid Raft |
| PMN | 1.0 | 1.0 |
| Cholesterol-depleted PMN | 0.8 | 0.7 |

**RESULTS**

Preliminary results show a consistent decrease in cholesterol content in cholesterol-depleted PMNs, with a decrease in phospholipid content observed to a lesser extent. Further studies are ongoing to validate these findings and to determine the specific role of cholesterol in the regulation of Rho GTPases.
LPS-induced Cdc42 Activation Occurs in Neutrophil Rafts

Fig. 1. Effect of LPS on Cdc42 and Rac activation. A, human PMNs (20 × 10^6/ml) were preincubated with 10 μM SB203580 or 0.1% MeSO carrier, treated with 100 ng/ml of E. coli 0111:B4 LPS for the times indicated, lysed, and then incubated with 5 μg of PBD-GST-agarose (4°C, 60 min). Precipitates were eluted, run on a 12% SDS-PAGE gel, transferred to nitrocellulose, and probed with either mouse anti-Rac or rabbit anti-Cdc42 antibody. Before incubation with PBD-GST, non-LPS-treated negative and positive control PMN lysates were treated with 1 mM GDP and 100 μM GTPγS, respectively. Lysates from all conditions were probed for total Cdc42 and Rac. The results shown are representative of five separate experiments. B, using the same methods as in A, activation of Rac by 10 μM fMLP was assayed. A representative experiment of three consecutive experiments is shown.

RESULTS

LPS Activates Cdc42 by a p38-dependent Mechanism— Whereas an important role for Rho GTPases in such prototypical inflammatory responses of the PMN as O_2\textsuperscript{2} generation and actin polymerization has been demonstrated previously (16, 17), to our knowledge, there have been no reports of activation of Rho GTPases by LPS in the human PMN. Given that LPS activates only the p38 MAPK arm of the MAPK superfamily in the human PMN in suspension (33), and that p38 plays an important role in O_2\textsuperscript{2} generation (18), we further queried whether p38 may lie upstream of Cdc42. In order to test these hypotheses, affinity precipitation was performed upon lysates of LPS-exposed PMNs using a recombinant PBD-GST fusion protein (19), which recognizes only the active, GTP-bound form of Cdc42 and Rac. Immunoblotting of the affinity precipitates indicates that LPS induces a time-dependent increase in the abundance of GTP-bound Cdc42 first detected after 5 min (Fig. 1A) and observed for 40 min (data not shown). LPS-induced activation of Cdc42 is inhibited by preincubation with the p38 inhibitor SB203580. No consistent effect upon LPS-induced Cdc42 activation was noted with piceatannol, a tyrosine kinase inhibitor, nor wortmannin, a phosphatidylinositol 3-kinase inhibitor (data not shown). Because Cdc42 activation by TLR2 agonists such as S. aureus has been reported previously in THP-1 cells (15), and concern has been expressed over the possibility of TLR2 agonists contaminating commercial LPS preparations (34), we tested whether peptidoglycan, a TLR2 agonist, would activate Cdc42 in the human PMN. Similar to S. aureus in THP-1 cells (15), peptidoglycan induce a rapid and transient activation of Cdc42 in the human PMN, with activation noted at 1-2 min and a return to base line before 5 min (data not shown). The much more delayed and prolonged time course of Cdc42 activation noted with LPS (Fig. 1) indicates that TLR2 agonist contaminants (e.g. bacterial lipoproteins) are very unlikely to explain the phenomena noted with LPS in this study.

LPS does not induce activation of Rac in the human PMN (Fig. 1A). Whereas no Rac activation was noted with LPS exposures ranging between 1 and 30 min (data not shown), rapid and transient Rac activation was observed after fMLP treatment (Fig. 1B). Of note, Rac activation in the human PMN has been reported previously not only with fMLP but also with platelet-activating factor, phorbol myristate acetate, and leukotriene B_4 (35, 36).
LPS Induces Recruitment of Active Cdc42 to, and Exclusion of Rac from, Lipid Rafts

To test the possibility that LPS activates Cdc42 in human PMN rafts, PMNs were left untreated or exposed to LPS for 15 min. Following this, the cells were lysed, and 10 contiguous sucrose density gradient fractions of the lysates were collected as described under “Experimental Procedures.” Twenty μg of protein from all fractions (Bradford protein assay (21)) were tested for GTP-Cdc42 by incubation with 5 μg of PBD-GST-agarose (4 °C, 60 min) followed by SDS-PAGE, transfer to nitrocellulose, and probing with anti-Cdc42 antibody. Equal protein loads (1 μg) from all fractions were also probed for total Cdc42 and flotillin-1. Fractions were tested for alkaline phosphatase (AP) activity (see “Experimental Procedures”). B, LPS induces translocation of signaling proteins to and from rafts. PMNs were left untreated or incubated with 100 ng/ml E. coli 0111:B4 LPS for the times indicated, and rafts were isolated (see “Experimental Procedures”). Rafts from each time point were identified by alkaline phosphatase activity in two contiguous low density fractions (fractions 8 and 9), and 10 μg of protein from these fractions was electrophoresed on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies of interest. C, LPS activates signaling proteins in rafts. Raft fractions from LPS-exposed PMNs were probed with anti-PO_{4}-p38. To confirm a time course of Cdc42 activation in lipid rafts, PBD-GST-agarose precipitates from raft fractions were probed with anti-Cdc42. The results shown are representative of three separate experiments.

LPS Induces Recruitment of Active Cdc42 to, and Exclusion of Rac from, Lipid Rafts — To test the possibility that LPS activates Cdc42 in human PMN rafts, PMNs were left untreated or exposed to LPS for 15 min. Following this, the cells were lysed, and 10 contiguous sucrose density gradient fractions were collected. Equal protein loads from all fractions were assayed for GTP-Cdc42 by incubation with PBD-GST-agarose, followed by elution, SDS-PAGE, transfer to nitrocellulose, and probing with anti-Cdc42. All fractions were also probed for total Cdc42 and were assayed for the presence of rafts by measurement of alkaline phosphatase activity and by immunoblotting with anti-flotillin-1 antibody. Of interest, despite the fact that the vast majority of cellular Cdc42 is localized to non-raft fractions in both nonstimulated and LPS-treated PMNs, Cdc42 is selectively activated in raft fractions, as identified by flotillin-1 (Fig. 2A). This suggests that LPS activates a very minor, raft-localized, subfraction of total cellular Cdc42 to high specific activity. The small degree of Cdc42 activation in nonstimulated cells is also localized to rafts (Fig. 2A). LPS alters neither the profile of alkaline phosphatase activity nor flotillin-1 abundance among sucrose density fractions (Fig. 2A).

To confirm that LPS induces dynamic recruitment of total Cdc42 to lipid rafts, as has been demonstrated previously (37) for other signaling proteins, the lipid raft microdomain (collected from fractions 8 and 9, corresponding to Fig. 2A) was isolated from human PMNs exposed to LPS for varying durations ranging from 2 to 20 min. A 10-fold higher protein load (10 μg) than that immunoblotted from each fraction in Fig. 2A
Cholesterol Depletion Activates p38 and Cdc42

**LPS-induced Cdc42 Activation Occurs in Neutrophil Rafts**

Cyclodextrins are cyclic oligomers of glucose that extract cholesterol from intact, living cells in a reversible, time- and concentration-dependent fashion (40). Their utility lies in the fact that cyclodextrin-mediated cellular cholesterol depletion is thought to disrupt lipid rafts physically and functionally (41), thereby providing clues to the native regulatory influence of intact rafts upon cellular phenomena. Moreover, the specificity for raft integrity of cyclodextrin-induced effects can be confirmed by using the cyclodextrin-cholesterol complex as a vehicle subsequently to “replete” cellular cholesterol in cyclodextrin-depleted cells, thereby restoring raft integrity and function (5). Because several different cyclodextrin compounds and protocols have been applied in the literature, and the specificity of cyclodextrins for raft versus non-raft cholesterol is a matter of some dispute (4, 27), we preferred to confirm the specificity of our cholesterol depletion protocol for PMN raft microdomains.

Rather than quantitating absolute cholesterol levels in membrane fractions as others have done (5), we normalized cholesterol to membrane phospholipid content in an attempt to verify the specificity of cyclodextrin for cholesterol extraction. As depicted in Fig. 3A, our mβCD depletion protocol (5 mM mβCD, 15 min, 37 °C) extracts cholesterol specifically from rafts. Nevertheless, our mβCD protocol did not noticeably alter the distribution of CD55, flotillin, or alkaline phosphatase activity within sucrose density gradients of resting PMNs (data not shown, see “Discussion”).

Like other investigators, we tested the reversibility of effects induced by cholesterol depletion by performing cholesterol repletion on depleted cells. This sequential repletion (see below and under “Experimental Procedures”) is thought largely to restore raft structure and function. However, we surmised that certain physiologic effects of mβCD-mediated cholesterol depletion described below (e.g. actin polymerization) would be unlikely to be reversed upon subsequent cholesterol repletion, despite effective re-ordering of rafts. Therefore, we sought to confirm a dose of cholesterol complexed to mβCD for primary treatment of cells that could serve as a control for the raft specificity of mβCD by maintaining cellular cholesterol at a constant level, what we have termed iso-repletion. As depicted

in Fig. 3B, 10 µM cholesterol complexed with mβCD was confirmed to maintain base-line cellular cholesterol levels. Progressive increases in the amount of cholesterol complexed to mβCD yield progressively higher deliveries of cholesterol to the cell (data not shown). In sum, these studies confirm the selectivity of the mβCD depletion protocol for raft cholesterol.

**Cholesterol Depletion Activates p38 and Cdc42**—Given our observations of the raft localization of p38, Cdc42, and Rac, and of LPS-induced translocation of the latter two (Fig. 2A), we queried whether rafts might exert a regulatory influence upon the activation state of these signaling proteins. To address this question, we performed immunoblotting upon lysates (to quan-
titate active phospho-p38) and PBD-GST affinity precipitates (to quantitate GTP-Cdc42 and-Rac) of PMNs that were cholesterol-depleted with mβCD or-iso-repleted with mβCD-10 μM cholesterol complex. As depicted in Fig. 4A, cholesterol depletion induces phosphorylation of p38 (phosphospecific antibody immunoblot), Cdc42, and Rac (anti-Cdc42 and anti-Rac immunoblots of PBD-GST-agarose affinity precipitates) was determined. B, effect of p38 inhibition upon Cdc42 activation induced by cholesterol depletion. An aliquot of PMNs was preincubated with 10 μM SB203580 (37°C, 15 min) before incubation with mβCD and then assayed for GTP-Cdc42 as described above. C, effect of cholesterol depletion upon LPS-induced activation of p38 and Cdc42. PMNs were either left untreated, depleted with 5 mM mβCD, depleted with mβCD and then sequentially cholesterol-repleted, or depleted with mβCD and then incubated with mβCD-pregnenolone, a complex ineffective at cholesterol repletion. All four conditions were then left untreated or incubated with 100 ng/ml E. coli LPS (20 min). The activation state of p38 and Cdc42 was determined by immunoblotting, as described above. The data shown are representative of three separate experiments.

LPS-induced Cdc42 Activation Occurs in Neutrophil Rafts

FIG. 4. Effect of cholesterol depletion upon p38, Cdc42, and Rac activation. A, effect in nonstimulated PMNs. PMNs were either left untreated, cholesterol-depleted with 5 mM mβCD, or cholesterol-iso-repleted with mβCD-10 μM cholesterol complex (37°C, 15 min). The activation state of p38 (phosphospecific antibody immunoblot), Cdc42, and Rac (anti-Cdc42 and anti-Rac immunoblots of PBD-GST-agarose affinity precipitates) was determined. B, effect of p38 inhibition upon Cdc42 activation induced by cholesterol depletion. An aliquot of PMNs was preincubated with 10 μM SB203580 (37°C, 15 min) before incubation with mβCD and then assayed for GTP-Cdc42 as described above. C, effect of cholesterol depletion upon LPS-induced activation of p38 and Cdc42. PMNs were either left untreated, depleted with 5 mM mβCD, depleted with mβCD and then sequentially cholesterol-repleted, or depleted with mβCD and then incubated with mβCD-pregnenolone, a complex ineffective at cholesterol repletion. All four conditions were then left untreated or incubated with 100 ng/ml E. coli LPS (20 min). The activation state of p38 and Cdc42 was determined by immunoblotting, as described above. The data shown are representative of three separate experiments.

CD14 and other members of the LPS receptor complex have been reported to reside within the lipid raft microdomain of monocytes (9, 39). We therefore further queried whether cholesterol depletion would inhibit subsequent LPS-induced acti-
Lipid rafts are cholesterol-rich membrane microdomains that are thought to organize agonist-induced interactions among signaling proteins (1, 2). They have been demonstrated to play an important regulatory role in multiple signaling pathways, including B and T cell receptor signaling, PDGF signaling, and most recently, LPS signaling. As a physicochemically isolable signaling compartment, rafts have provided a new window upon novel and sometimes unexpected signaling interactions.

By embarking upon previous reports of dynamic translocation of Rho family GTPases to rafts (11), and of a role for these proteins in the LPS signaling cascade in cell lines (13, 14), we report that LPS activates Cdc42 by a p38-dependent mechanism in the human PMN and induces translocation of Cdc42 to PMN rafts. Raft-associated Cdc42 and p38 are found to be activated after LPS exposure. We confirm the presence of the LPS receptor component, CD14, within human PMN rafts. Moreover, selective perturbation of raft function with mβCD suggests a role for rafts in both 1) the maintenance of p38 and Cdc42 in their inactive form in the resting human PMN, and 2) the transmission of the LPS signal to both p38 and Cdc42. Regarding the former, a previous report (47) describing no activation of p38 with mβCD treatment of NIH 3T3 cells suggests cell type specificity for this phenomenon. A report of ligand-independent activation of the epidermal growth factor receptor by mβCD (48) suggests a possible mechanism, by analogy, for the recapitulation by mβCD of multiple effects of LPS noted in the present study (i.e. activation of p38 and Cdc42 but not Rac; O2− priming; and actin polymerization). However, this phenomenon is unlikely to apply to the LPS receptor because mβCD activates extracellular signal-regulated kinase in the human PMN (data not shown) but LPS does not (33). Furthermore, mβCD reversibly primes for O2− release (Fig. 5) and remodels actin in a clearly distinct manner from LPS (Fig. 6B). Regarding a role for rafts in transmission of the LPS signal to p38, it has been reported previously (9) that tumor necrosis factor-α secretion by LPS-stimulated human monocytes is blocked by preincubation with mβCD. Most interestingly, raft integrity appears to be somewhat specific for transmission of the LPS signal to p38, because mβCD pretreatment does not inhibit hyperosmotic shock-induced p38 phosphorylation (47).

Cdc42 is activated selectively in PMN rafts, whereas p38 is activated both within and outside PMN rafts. Although we are not aware of previous reports of p38 within lipid rafts, a precedent for raft compartmentalization of p38 activity is provided by one report that p38 inhibition decreases stress-induced phosphorylation of the raft-localized protein caveolin-1 in NIH 3T3 cells (47). LPS has also been reported to activate c-Jun phosphorylation and Cdc42 activation. Inhibition of LPS-induced p38 activation by cholesterol depletion was also confirmed with an in vitro kinase assay (data not shown). Because of the high degree of activation of p38 and Cdc42 induced by cholesterol depletion of depleted cells, firm conclusions are not possible regarding the restoration of the LPS effect with sequential cholesterol repletion (Fig. 4B).

Cholesterol Depletion Primers for Superoxide Anion (O2−) Release—Rac plays a central regulatory role in O2− production (17). LPS is an effective priming agent for subsequent O2− release triggered by such agents as the formylated bacterial tripeptide fMLP (42). Given these premises, and our observation of LPS-induced translocation of Rac out of rafts (Fig. 2), we queried whether rafts might exert a regulatory influence upon O2− generation in the human PMN. We therefore tested whether mβCD has any priming effect upon subsequent O2− production triggered by fMLP (30). As depicted in Fig. 5, cholesterol depletion with mβCD was effective by itself in priming the PMN for subsequent O2− production triggered by both fMLP and cytochalasin D, a commonly used PMN priming agent (43).

Whereas this priming effect of mβCD upon fMLP-induced O2− production is in agreement with one previous report (44), to our knowledge ours is the first report to confirm the reversibility of this priming effect with sequential cholesterol repletion (Fig. 5), but not with the pregnenolone control. Reversal of priming was also observed with sequential treatment with 5-cholenol-3-one, an alternative molecule effective in cellular cholesterol repletion (Fig. 5).

Cholesterol Depletion Induces Actin Polymerization—Because Cdc42 is thought to play an important role in actin remodeling (45) and is regulated by lipid rafts (Fig. 4), we queried whether rafts might regulate actin polymerization in the human PMN. As shown in Fig. 6A, cholesterol depletion is, by itself, a potent stimulus for actin polymerization in the human PMN, inducing actin assembly to a magnitude comparable with LPS. We thought it unlikely that subsequent cholesterol repletion of the mβCD-treated cells would reverse F-actin formation and thereby serve as a suitable control to demonstrate the raft specificity of the mβCD effect. In fact, sequential repletion of cholesterol-depleted cells was noted to further increase F-actin content beyond that measured with mβCD treatment (data not shown), suggesting that perturbations of cellular cholesterol content upward and downward are both sufficient to induce actin polymerization. Most intriguingly, in this vein, we noted a parallel increase in both p38 phosphorilation and Cdc42 activation (Fig. 4C) with sequential repletion over that observed with initial cholesterol depletion. As shown in Fig. 6A, the raft specificity of mβCD-mediated actin polymerization was instead demonstrated by effective blockade of mβCD-induced actin polymerization by the inclusion of cholesterol in an iso-repletion protocol. Confocal microscopy of PMNs stained with rhodamine-phalloidin indicates that LPS and mβCD induce markedly different morphologies of F-actin remodeling, the former a pattern of cytoskeletal polarization, as reported previously (46), and the latter a cortical rim of F-actin (Fig. 6B).

**DISCUSSION**

Lipid rafts are cholesterol-rich membrane microdomains that are thought to organize agonist-induced interactions among signaling proteins (1, 2). They have been demonstrated to play an important regulatory role in multiple signaling pathways, including B and T cell receptor signaling, PDGF signaling, and most recently, LPS signaling. As a physicochemically isolable signaling compartment, rafts have provided a new window upon novel and sometimes unexpected signaling interactions.

By embarking upon previous reports of dynamic translocation of Rho family GTPases to rafts (11), and of a role for these proteins in the LPS signaling cascade in cell lines (13, 14), we report that LPS activates Cdc42 by a p38-dependent mechanism in the human PMN and induces translocation of Cdc42 to PMN rafts. Raft-associated Cdc42 and p38 are found to be activated after LPS exposure. We confirm the presence of the LPS receptor component, CD14, within human PMN rafts. Moreover, selective perturbation of raft function with mβCD suggests a role for rafts in both 1) the maintenance of p38 and Cdc42 in their inactive form in the resting human PMN, and 2) the transmission of the LPS signal to both p38 and Cdc42. Regarding the former, a previous report (47) describing no activation of p38 with mβCD treatment of NIH 3T3 cells suggests cell type specificity for this phenomenon. A report of ligand-independent activation of the epidermal growth factor receptor by mβCD (48) suggests a possible mechanism, by analogy, for the recapitulation by mβCD of multiple effects of LPS noted in the present study (i.e. activation of p38 and Cdc42 but not Rac; O2− priming; and actin polymerization). However, this phenomenon is unlikely to apply to the LPS receptor because mβCD activates extracellular signal-regulated kinase in the human PMN (data not shown) but LPS does not (33). Furthermore, mβCD reversibly primes for O2− release (Fig. 5) and remodels actin in a clearly distinct manner from LPS (Fig. 6B). Regarding a role for rafts in transmission of the LPS signal to p38, it has been reported previously (9) that tumor necrosis factor-α secretion by LPS-stimulated human monocytes is blocked by preincubation with mβCD. Most interestingly, raft integrity appears to be somewhat specific for transmission of the LPS signal to p38, because mβCD pretreatment does not inhibit hyperosmotic shock-induced p38 phosphorylation (47).

Cdc42 is activated selectively in PMN rafts, whereas p38 is activated both within and outside PMN rafts. Although we are not aware of previous reports of p38 within lipid rafts, a precedent for raft compartmentalization of p38 activity is provided by one report that p38 inhibition decreases stress-induced phosphorylation of the raft-localized protein caveolin-1 in NIH 3T3 cells (47). LPS has also been reported to activate c-Jun phosphorylation and Cdc42 activation. Inhibition of LPS-induced p38 activation by cholesterol depletion was also confirmed with an in vitro kinase assay (data not shown). Because of the high degree of activation of p38 and Cdc42 induced by cholesterol depletion of depleted cells, firm conclusions are not possible regarding the restoration of the LPS effect with sequential cholesterol repletion (Fig. 4B).

**Cholesterol Depletion Primers for Superoxide Anion (O2−) Release—**Rac plays a central regulatory role in O2− production (17). LPS is an effective priming agent for subsequent O2− release triggered by such agents as the formylated bacterial tripeptide fMLP (42). Given these premises, and our observation of LPS-induced translocation of Rac out of rafts (Fig. 2), we queried whether rafts might exert a regulatory influence upon O2− generation in the human PMN. We therefore tested whether mβCD has any priming effect upon subsequent O2− production triggered by fMLP (30). As depicted in Fig. 5, cholesterol depletion with mβCD was effective by itself in priming the PMN for subsequent O2− production triggered by both fMLP and cytochalasin D, a commonly used PMN priming agent (43).

Whereas this priming effect of mβCD upon fMLP-induced O2− production is in agreement with one previous report (44), to our knowledge ours is the first report to confirm the reversibility of this priming effect with sequential cholesterol repletion (Fig. 5), but not with the pregnenolone control. Reversal of priming was also observed with sequential treatment with 5-cholenol-3-one, an alternative molecule effective in cellular cholesterol repletion (Fig. 5).

**Cholesterol Depletion Induces Actin Polymerization—**Because Cdc42 is thought to play an important role in actin remodeling (45) and is regulated by lipid rafts (Fig. 4), we queried whether rafts might regulate actin polymerization in the human PMN. As shown in Fig. 6A, cholesterol depletion is, by itself, a potent stimulus for actin polymerization in the human PMN, inducing actin assembly to a magnitude comparable with LPS. We thought it unlikely that subsequent cholesterol repletion of the mβCD-treated cells would reverse F-actin formation and thereby serve as a suitable control to demonstrate the raft specificity of the mβCD effect. In fact,
production and actin polymerization. A, PMNs were either left untreated (buffer), incubated with 100 ng/ml E. coli LPS (45 min), incubated with 5 mM mβCD, or cholesterol-iso-repleted with mβCD/10 μM cholesterol (chol). F-actin was then assayed by cell fixation/permeabilization followed by staining with NBD-phalloidin and flow cytometry (31). Actin assembly was quantified by the relative fluorescence index (RFI) or mean fluorescence of the sample divided by that of nonstimulated cells. The values (±S.E.) shown were generated from three separate experiments. B, PMNs were either left untreated, incubated with LPS, or incubated with mβCD, as described above in A, and then fixed, permeabilized, and stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR). Photographs were taken using an Olympus Vanox Microscope with 60x oil objective using Slidebook software (Intelligent Imaging, Denver, CO).

NH$_2$-terminal kinase both within and outside rafts in Mono-Mac-6 cells (9), and mβCD has been reported to activate extracellular signal-regulated kinase within COS-1 cell rafts and to prime for epidermal growth factor-induced activation of extracellular signal-regulated kinase in COS-1 lysates (4). Activation and translocation of Rac1 and RhoA to fibroblast rafts have been reported to be induced by PDGF (12). Consistent with these reports on other Rho GTPases, the present work indicates that PMN rafts play an important role in Cdc42 regulation. This is evinced both by the selective, raft-localized activation of Cdc42 induced by LPS and by detectable increases in GTP-Cdc42 from lysates of mβCD-treated cells compared with untreated cells (Fig. 4A). We found that mβCD activates p38 and Cdc42 in resting PMNs and yet inhibits LPS-induced activation of p38 and Cdc42 (Fig. 4). We speculate that these findings can be reconciled by the hypothesis that resting PMN rafts exert a negative regulatory influence upon Cdc42, and yet also contain feed-forward positive signals important to LPS-induced activation of Cdc42.

The similar temporal and quantitative increases of total Cdc42 and GTP-Cdc42 in rafts induced by LPS (Fig. 2) suggest that most, if not all, raft-localized Cdc42 is activated, as has been suggested for other Rho GTPases (12). This, in turn, is consistent with either one of two mechanistic possibilities: 1) activation of Cdc42 induces its translocation to lipid rafts or 2) translocation of Cdc42 to lipid rafts induces its activation in that location. These two mechanisms are not distinguished by the present study. The first is supported by a previous report (12) that GTPγS treatment of fibroblast lysates induces translocation of RhoA to caveolae. The second is supported by our observation of the co-localization within rafts of two positive Cdc42 regulators, p38 and moesin, the latter reported to activate Cdc42 by exclusion of Rho GDP-dissociation inhibitor (38). Of interest, moesin has been reported previously to link lipid rafts with the actin cytoskeleton in T lymphocytes (49), to be a potential receptor for LPS on monocytes (50), and consistent with our findings (Fig. 2B), to further translocate to the PMN plasma membrane upon activation (51).

In addition to demonstrating a role for rafts in regulation of Cdc42 activity, we also demonstrate raft regulation of two Rho GTPase-dependent functions, O$_2^*$ production and actin polymerization. Most interestingly, cholesterol depletion was a potent, yet reversible priming mechanism for fMLP-triggered O$_2^*$ production (Fig. 5). Although the mechanism underlying this phenomenon remains unclear, it cannot be explained solely by mβCD-induced activation of p38 or Cdc42. fMLP-induced O$_2^*$ release is p38-dependent in the PMN (18). However, sequential cholesterol repletion of depleted cells increases p38 phosphorylation, yet reverses mβCD-induced O$_2^*$ priming (Figs. 4C and 5). Similarly, whereas Cdc42, like Rac, has been linked to the oxidative burst of the PMN (52), sequential repletion also increases its activation over that observed with initial cholesterol depletion (Fig. 4C). Like mβCD, LPS primes the human PMN for fMLP-triggered O$_2^*$ production without activating Rac (Figs. 1, 4, and 5). Whereas the mechanism of both mβCD- and LPS-induced O$_2^*$ priming remains somewhat unclear in the literature, it is tempting to speculate that they both may operate through relocalization of Rac away from the raft compartment (Fig. 2B) or, potentially, through relocalization of other proteins, such as the Phox components.

mβCD induces actin polymerization in a subcellular distribution markedly distinct from that induced by LPS (Fig. 6B). Of interest, this pattern of actin remodeling is very similar to that noted in the PMN within the first 30 s of fMLP exposure (53), suggesting that mβCD and fMLP may operate through like mechanisms. The comparison is, however, complicated by the fact that fMLP is reported to activate RhoA, Cdc42, and Rac (the last through a Cdc42-dependent mechanism) in the human PMN (54, 55). Unlike O$_2^*$ production and most other physiologically relevant functions of the LPS-stimulated human PMN (e.g. tumor necrosis factor-α production, adhesion, and chemotaxis), actin polymerization in the human PMN is p38-independent (33). This suggests that LPS-induced activation of Cdc42 is not central to, or at least not a rate-limiting step in, the remodeling of actin produced by LPS. Future studies will need to test whether LPS and/or mβCD activate RhoA.
Limitations of the present study should be noted. Although we did not directly measure intrinsic GTPase activity, quantitation of GTP-Cdc42 with PBD affinity precipitation is a well accepted and reliable technique (19). The terminally differentiated human PMN cannot be transfected, thus limiting studies such as the present one to the use of chemical inhibitors. Although some controversy exists regarding the relationship between “detergent-resistant membranes,” Triton-insoluble low density membrane fractions, as isolated in the present study, and “lipid rafts,” this nevertheless represents a well accepted methodology for raft isolation (20). The demonstration of two proteins in raft isolates does not prove their physical association. In fact, rafts are found not only in the plasma membrane but also within intracellular membranes (1, 2), and rafts from both locations are expected to be collected together by our methodology. The significance of a broader distribution of rafts within the sucrose density gradient identified by flotillin-1 as compared with alkaline phosphatase activity (Fig 24) remains somewhat uncertain; nevertheless, flotillin-1 is an integral membrane protein that has been confirmed to be raft-specific in multiple cells, including PMNs (22, 23). Moreover, we confirmed both translational of total Cdc42 to, and activation of Cdc42 in, raft fractions identified by the narrower range of alkaline phosphatase activity (Fig 2C). In any event, several groups have reported the existence of heterogeneous subtypes of lipid rafts within single cell types, and in fact, one group, like us, has reported previously (56) that raft-localized Cdc42 has a slightly denser distribution compared with other raft markers such as CD55 and CD14.

Regarding the results derived from cholesterol depletion, we cannot exclude the possibility that we may have obtained different results with a different depletion protocol, such as one using a different cyclodextrin compound or extracting a higher percentage of cellular cholesterol. Comparable or less aggressive cholesterol depletion protocols have been validated previously for the neutrophil (57, 58). Although some groups have variably for the neutrophil (57, 58). Although some groups have variably for the neutrophil (57, 58). Although some groups have variably for the neutrophil (57, 58). Although some groups have variably for the neutrophil (57, 58). Although some groups have variably for the neutrophil (57, 58). Although some groups have variably for the neutrophil (57, 58). Although some groups have variably for the neutrophil (57, 58). Although some groups have variably for the neutrophil (57, 58).

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LPS-induced Cdc42 Activation Occurs in Neutrophil Rafts
Lipid Rafts Regulate Lipopolysaccharide-induced Activation of Cdc42 and Inflammatory Functions of the Human Neutrophil

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