Macrophages respond to Gram-negative bacterial pathogens by phagocytosis and pro-inflammatory gene expression. These responses may require GTPases that have been implicated in cytoskeletal alterations and activation of NF-κB. To determine the role of Rac1 and Cdc42 in signal transduction events triggered by *Pseudomonas aeruginosa*, we expressed GTP binding-deficient alleles of Rac1 or Cdc42, or Chim-GAP, a Rac1/Cdc42-specific GTPase-activating protein domain, in a subline of RAW 264.7 cells, and challenged the transfected cells with a laboratory strain of *P. aeruginosa*, PAO1. Expression of Rac1 N17, Cdc42 N17, or Chim-GAP led to a marked reduction of phagocytosis. In contrast, nuclear translocation of p65 NF-κB was unaffected by expression of the same constructs. Incubation of macrophages with PAO1 led to NF-κB-dependent expression of inducible nitric-oxide synthase, COX-2, and tumor necrosis factor-α, which was unaffected by inhibition of Rac1 or Cdc42 function. Isogenic strains of PAO1 that lacked surface adhesins were poorly ingested; however, they induced pro-inflammatory gene expression with an efficiency equal to that of PAO1. These results indicate that the signal transduction events leading to phagocytosis and pro-inflammatory protein expression are distinct. Rac1 and Cdc42 serve as effectors of phagocytosis, but not NF-κB-dependent gene expression, in the macrophage response to *P. aeruginosa*.

Phagocytic leukocytes, such as macrophages and polymorphonuclear leukocytes, respond to bacterial pathogens by the process of phagocytosis, an early and essential step in the leukocyte bactericidal response. Bacterial ingestion is accompanied by expression of pro-inflammatory gene products, which is a major mechanism utilized by phagocytes to orchestrate an anti-bacterial immune response. Although the relationship between phagocytosis and gene expression is uncertain, several studies have suggested that phagocytosis per se triggers gene expression (1–7). Whether phagocytosis and its underlying cytoskeletal alterations directly contribute to gene expression is unclear, but the ability of several phagocytosis-promoting receptors to trigger the activation of NF-κB suggests one way in which phagocytosis may contribute to gene expression (8–10). In addition, phagocytosis of IgG-coated particles (11–13) and Salmonella typhimurium (14) requires the participation of Rac1 and Cdc42, GTPases that trigger cytoskeletal alterations and have the capacity to activate transcriptional pathways, including AP-1, via c-Jun N-terminal kinase (15), and NF-κB (16, 17). Furthermore, Rac is a component of the NADPH oxidase (18), which produces superoxide anion upon activation, leading to the accumulation of other reactive oxygen intermediates. One or more of these reactive compounds may serve to activate transcriptional pathways, including NF-κB, in vivo (19).

*Pseudomonas aeruginosa* is a Gram-negative bacterium that causes infections in immunocompromised hosts, such as individuals with cystic fibrosis, burn victims, and patients infected with human immunodeficiency virus (for review, see Ref. 20). The interaction of *Pseudomonas* with macrophages occurs via multiple cell surface receptors, and is accompanied by the formation of pseudopods that resemble those that arise during Fcγ receptor (FcγR)1-mediated phagocytosis (21). However, the role of the actin-based cytoskeleton and the signal transduction mechanisms that govern phagocytosis of *Pseudomonas* are unknown, and the relationship between phagocytosis and gene expression is unclear. In this study, we investigated the role of Rac1 and Cdc42 in the phagocytosis of *P. aeruginosa* by macrophages, and determined whether these GTPases contributed to the production of COX-2, iNOS, and TNF-α, gene products that contain multiple κB enhancer sites in their promoters. We addressed the question whether phagocytosis, or signal transduction events that underlie it, are required for NF-κB-dependent gene expression.

**Experimental Procedures**

**Cells and Reagents**—RAW LR/FMLPR 2 cells (11), a subline of the RAW 264.7 murine macrophage-like cell line (22), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin and maintained at 37 °C in a 5% CO2 incubator. Myc-tagged Rac1 N17, Cdc42 N17, or the GAP domain of n-chimaerin (Chim-GAP) subcloned into pCMV3Rluc, were used for transfections as described previously (11). Myc-tagged Rac1 L61 and Cdc42 L61 subcloned in pRK5 (23) were kindly provided by Dr. Alan Hall (University College London, United Kingdom). A plasmid containing IκBα was kindly provided by Dr. Dimitrios Thanos (Columbia University, New York, NY). The following isogenic strains of *P. aeruginosa* were maintained on LB agar plates supplemented with 50 μg/ml ampicillin: FAO1, PA 340 (Pil^{-} F116r PO4^{-}) (Ref. 24) and AK1152 (Pil+ Mot+; Ref. 25), and PA 477 (Pil+ F1α+); provided by Dr. **

1 The abbreviations used are: FcγR, receptor for the Fc portion of IgG; AMCA, aminomethylcoumarin; AP-1, activator protein-1; GAP, GTPase-activating protein; Chim-GAP, the GTPase-activating protein domain of n-chimaerin; COX-2, cyclooxygenase-2; FITC, fluorescein isothiocyanate; iNOS, inducible nitric-oxide synthase; TNF, tumor necrosis factor; mAb, monoclonal antibody; CFU, colony-forming unit(s); GEF, guanine nucleotide exchange factor; LPS, lipopolysaccharide.
Alice Prince, Columbia University, New York, NY). Rabbit serum against PAO1 was a gift from Dr. Alice Prince. A mouse mAb against the Myc epitope was from Roche Molecular Biochemicals. Rabbit IgG (C-20) against p65 NF-κB, mouse mAb (H-4) against IκBα, and rabbit IgG (M-19) against iNOS were from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit IgG against COX-2 was from Cayman Chemical Co. (Ann Arbor, MI). Rat IgG against TNF-α was from PharMingen (San Diego, CA). Rhodamine- and FITC-conjugated anti-rabbit IgG, FITC-conjugated anti-mouse IgG, AMCA-conjugated anti-mouse IgG, biotin-conjugated anti-rabbit and anti-goat IgG, and horseradish peroxidase-conjugated streptavidin were from Jackson ImmunoResearch (West Grove, PA). Rhodamine-conjugated streptavidin and fluorescein-phalloidin were from Molecular Probes (Eugene, OR).

**Bacterial Association and Phagocytosis Assays**—Sixteen hours following transfection of plasmids encoding the indicated constructs, adherent RAW LR/FMLPR.2 cells were incubated with 4.5 × 10⁵ CFU PAO1 for 30 min at 37 °C, followed by washing non-adherent bacteria and incubation with PAO1 antiserum at 4 °C (to detect bound, unengulfed bacteria), followed by FITC-conjugated anti-rabbit IgG. The cells were subsequently fixed in 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and stained with PAO1 antiserum, followed by rhodamine-conjugated anti-rabbit IgG to detect all cell-associated bacteria. Stained cells were visualized using fluorescence microscopy and scored for the presence of attached, but unengulfed (green) and total (red) bacteria. Ingested bacteria represent the difference between total and attached, unengulfed bacteria (i.e. red minus green). Myc-expressing cells were identified using a mAb against Myc and AMCA-conjugated anti-mouse IgG. Data are presented as association index (number of PAO1 either bound to, or ingested by, 100 macrophages) and phagocytosis index (number of PAO1 ingested by 100 macrophages). A total of 100 Myc-expressing and 100 non-Myc-expressing cells, in at least 7 microscopic fields, were analyzed in each experiment, which was repeated three times.

**Assay for Nuclear Localization of p65 NF-κB**—Sixteen hours following transfection of plasmids encoding the indicated constructs, adherent macrophages were incubated in the presence or absence of 4.5 × 10⁵ CFU PAO1 for 30 min at 37 °C. Following fixation with 3.7% formaldehyde and permeabilization with 0.2% Triton X-100, cells were stained with anti-p65 NF-κB followed by rhodamine-conjugated anti-rabbit IgG to detect NF-κB and with either mAb anti-Myc followed by FITC-conjugated anti-mouse IgG to detect Myc-tagged proteins, or mAb anti-IκBα followed by FITC-conjugated anti-mouse IgG to detect those cells overexpressing IκBα. In some experiments, F-actin was visualized using fluorescein-phalloidin and Myc-tagged proteins were visualized using mAb anti-Myc followed by AMCA-conjugated anti-mouse IgG. Nuclear localization of p65 NF-κB was scored as “positive” if fluorescence was clearly visible over the nucleus, there was a clear demarcation between nuclear and cytoplasmic fluorescence, and the intensity of nuclear fluorescence exceeded that of the cytoplasm. A total of 50 Myc-expressing cells and 50 non-Myc-expressing cells, in at least 7 microscopic fields, were analyzed in each experiment, which was repeated three times.

**Stress Assays for Detection of iNOS, COX-2, and TNF-α Protein**—Sixteen hours following transfection of plasmids encoding the indicated constructs, adherent macrophages were incubated in the presence of absence of 4.5 × 10⁵ CFU PAO1 for 6 h at 37 °C. Following fixation with 3.7% formaldehyde and permeabilization with 0.2% Triton X-100, cells were stained with either rabbit IgG against iNOS or COX-2, or with goat IgG against murine TNF-α, followed by biotin-conjugated secondary antibodies and rhodamine-conjugated streptavidin. Myc expression was detected using a mAb against Myc and FITC-conjugated anti-mouse IgG. IκBα expression was detected as described above. Quantitation of iNOS, COX-2, and TNF-α protein was done by measuring cell-associated fluorescence using single-cell microspectrophotometry (11). Fluorescence values were corrected for nonspecific fluorescence by using either non-immune rabbit IgG for iNOS and COX-2, or a rat myeloma IgG, for TNF-α. The nonspecific fluorescence did not exceed 10% of the total fluorescence. Myc-expressing cells were selected using fluorescein optics in a random fashion and without knowledge of rhodamine intensity. Control cells that did not demonstrate Myc expression were selected from the same slides. A total of 30 Myc-expressing cells and 30 non-Myc-expressing cells, in at least 7 microscopic fields, were analyzed in each experiment, which was repeated three times.

**Detection of iNOS, COX-2, and TNF-α Protein by Immunoblotting**—Adherent RAW LR/FMLPR.2 macrophages (5 × 10⁵) were incubated in the absence or presence of the indicated number of bacteria for 5 h at 37 °C. For measurements of TNF-α, 50 μM brefeldin A was added to the cells to inhibit TNF-α secretion. Cells were subjected to detergent lysis and subjected to SDS-PAGE. After transfer to a nitrocellulose membrane, the membranes were incubated with rabbit polyclonal antibodies against iNOS, COX-2, and TNF-α, followed by horseradish peroxidase-conjugated secondary antibodies. The membranes were developed using enhanced chemiluminescence reagent, and the intensity of specific bands was quantitated using a densitometer.

**Results**

**Rac1 and Cdc42 Are Required for Phagocytosis of Unopsonized P. aeruginosa by Murine Macrophages**—Rac1 and Cdc42 have been implicated in phagocytosis of IgG-coated particles (11–13) and S. typhimurium (14). To determine whether these GTPases participate in the phagocytosis of P. aeruginosa, we expressed guanine nucleotide binding-deficient alleles of Rac1 or Cdc42, or a GFP for both proteins, in RAW LR/FMLPR.2 cells, and performed association and phagocytosis assays. Expression of any of these proteins resulted in a marked (83–92%) inhibition of the macrophage phagocytic capacity for PAO1. Expression of these same constructs resulted in a moderate (33–45%) inhibition of the total number of bacteria associated with the macrophages (Fig. 1). When expressed as percentage of ingestion, control macrophages ingested 66% of cell-associated bacteria while macrophages expressing any of these constructs ingested 9–12% of cell-associated bacteria. Phagocytosis was inhibited by 97 ± 0.7% in the presence of 2 μM cytochalasin D, demonstrating an essential role for actin polymerization in phagocytosis of P. aeruginosa. These results indicate that, similar to ingestion of IgG-coated erythrocytes and Salmonella, intact Rac1 and Cdc42 function is required for phagocytosis of unopsonized P. aeruginosa by murine macrophages.

**Lack of Requirement for Rac1 and Cdc42 in the Activation of NF-κB by P. aeruginosa in RAW LR/FMLPR.2 Cells**—Because transfection of RAW LR/FMLPR.2 cells results in a small percentage of cells expressing the gene of interest, we resorted to co-transfection of plasmids containing Rac1 and Cdc42 alleles with pCMV-κB reporter construct. However, the co-transfection efficiency of these cells proved to be variable, compelling us to utilize other means of assessing the state of NF-κB activation in transfected cells. Expression of activated alleles of Rac1 or Cdc42 leads to nuclear translocation and/or activation of NF-κB in COS-7 cells, NIH 3T3 cells (17), and Swiss-3T3 cells (26). To assess whether Rac1 and Cdc42 are capable of activating NF-κB in mouse macrophages, we transfected plasmids encoding either Myc-Rac1 L61 or Myc-

![FIG. 1. Phagocytosis of unopsonized P. aeruginosa by RAW LR/FMLPR.2 cells requires intact Rac1 and Cdc42 function.](image-url)
Cdc42 L61 in RAW LR/FMLPR.2 cells and assessed whether their expression influenced the nuclear localization of p65 NF-κB. Expression of either Myc-Rac1 L61 or Myc-Cdc42 L61 led to membrane ruffling or filopodia (Fig. 2A), consistent with previously published results (27). In either case, p65 NF-κB was localized to the cytoplasm and was particularly prominent in membrane ruffles in Rac1-transfected cells; there was no nuclear enrichment of p65 NF-κB in Myc-Rac1 L61- or Myc-Cdc42 L61-expressing cells. It is still possible that Rac1 or Cdc42, while insufficient to trigger activation of NF-κB directly, might be required for activation of NF-κB by other stimuli, including bacteria. Addition of PAO1 to adherent RAW LR/FMLPR.2 cells led to nuclear translocation of p65 NF-κB in nearly all cells. However, expression of Rac1 N17 (Fig. 2B and C), Cdc42 N17, or Chim-GAP (Fig. 2C) did not inhibit PAO1-induced nuclear translocation of p65 NF-κB. These results indicate that nuclear translocation of p65 NF-κB in RAW LR/FMLPR.2 cells in response to *P. aeruginosa* is independent of intact Rac1 and Cdc42 function.

**Expression of COX-2, iNOS, and TNF-α in Response to *P. aeruginosa***—Macrophages produce many pro-inflammatory proteins in response to bacterial products, including COX-2, iNOS, and TNF-α. The promoters for each of these contain multiple NF-κB enhancers, and use of reporter constructs or pharmacological inhibitors implicates activation of NF-κB in the pathway leading to expression of these proteins by multiple stimuli (for review, see Ref. 28). To determine whether expression of COX-2, iNOS, and TNF-α induced by *P. aeruginosa* requires the participation of NF-κB, we overexpressed IκBα, an inhibitory subunit of NF-κB that has been shown to inhibit NF-κB activation by a variety of stimuli (29, 30). Overexpression of IκBα in RAW LR/FMLPR.2 cells resulted in a decrease in the number of cells demonstrating nuclear localization of p65 NF-κB in response to PAO1 (Fig. 3A). Expression of IκBα also resulted in decreased expression of COX-2, iNOS, and TNF-α (Fig. 3B), which was most marked for iNOS and TNF-α. These data indicate that NF-κB is required for optimal expression of several pro-inflammatory proteins in RAW LR/FMLPR.2 cells in response *P. aeruginosa*.

**Rac1 and Cdc42 Are Not Required for Expression of iNOS, COX-2, and TNF-α in RAW LR/FMLPR.2 Cells Incubated with *P. aeruginosa***—Our data indicate that Rac1 and Cdc42 are incapable of autonomously activating nuclear translocation of p65 NF-κB in RAW LR/FMLPR.2 cells, and do not inhibit nuclear translocation of p65 NF-κB in response to PAO1. To determine whether intact function of either GTPase is required for pro-inflammatory protein expression, we expressed Rac1 N17, Cdc42 N17, or Chim-GAP in RAW LR/FMLPR.2 cells and measured expression of either COX-2, iNOS, and TNF-α in response to PAO1. To prevent secretion of TNF-α, we added brefeldin A to those cells in which TNF-α expression was measured. Expression of levels of Rac1 N17, Cdc42 N17, or Chim-GAP sufficient to markedly inhibit phagocytosis (Fig. 1) had no significant effect on expression of either COX-2 (Fig. 4A) or iNOS (Fig. 4B), and had a minor effect on production of TNF-α (Fig. 4C).

**Neither Bacterial Binding nor Ingestion Is Required for Induction of iNOS, COX-2, and TNF-α Production by Macrophages**—Pro-inflammatory gene expression induced by various bacteria has been ascribed to phagocytosis per se (1–4, 6, 7). However, the inability of inhibitors of Rac1 and Cdc42 function to inhibit gene expression, despite their inhibition of phagocytosis (Figs. 1 and 4) suggested that phagocytosis is not required for pro-inflammatory gene expression. To determine whether phagocytosis or bacterial adherence influenced expression of COX-2, iNOS, or TNF-α, we utilized mutant isogenic strains of *P. aeruginosa*.
and/or attachment of adhesin-deficient bacterial strains, all incubated with bacteria. Despite the lack of phagocytosis, Adherent RAW LR/FMLPR.2 cells transfected with a plasmid encoding IκBα to detect transfected cells. Nuclear localization of p65 NF-κB was determined for IκBα-expressing cells (hatched bars) and non-expressing controls (black bars) incubated in the absence or presence of PAO1. Data represent mean ± S.E., n = 3. The difference between nuclear localization of p65 NF-κB in PAO1-stimulated IκBα-expressing cells and non-expressing controls was statistically significant (p < 0.0001). B, transfected cells were incubated in the absence or presence of 4.5 × 10⁷ CFU PAO1 for 6 h at 37°C as described under “Experimental Procedures,” followed by fixation and staining for IκBα to detect transfected cells and for iNOS, COX-2, or TNF-α. Quantitation of protein expression was performed using microspectrofluorometry as described under “Experimental Procedures.” Data are depicted as fold increase in protein expression in cells incubated with PAO1 as compared with unstimulated controls in IκBα-expressing cells and non-expressing controls (black bars). Data represent mean ± S.E., n = 3. Differences between expression of COX-2, iNOS, and TNF-α in IκBα-expressing cells and non-expressing controls were statistically significant (p < 0.001, p < 0.05, and p < 0.0001, respectively).

PAO1 that lacked putative adhesins for leukocytes (31). PA 340, which lacks pilin (Pil−) adhered poorly to RAW LR/FMLPR.2 cells when compared with PAO1 (Fig. 5A). However, the absence of pilin did not prevent phagocytosis of those bacteria that did adhere to the macrophages (Fig. 5, A and B), indicating that the presence of pilin is required for adherence, but not phagocytosis, per se. In contrast, an intact flagellum was necessary for both binding and phagocytosis, since strain AK1152, which lacks flagellin (Fla−), and PA 477, which lacks both pilin and flagellin (Pil−/Fla−), were neither bound to, nor ingested by, RAW LR/FMLPR.2 cells (Fig. 5, A and B). These data confirm a requirement for an intact flagellum in phagocytosis of P. aeruginosa (31). We incubated adherent RAW LR/FMLPR.2 cells with PAO1, AK1152, or PA 477, and subjected the cells to detergent lysis and immunoblotting. We included the fungal metabolite brefeldin A in some samples to inhibit secretion of TNF-α. Immunoblotting revealed the presence of COX-2, iNOS, and the unprocessed form of TNF-α in cells incubated with bacteria. Despite the lack of phagocytosis and/or attachment of adhesin-deficient bacterial strains, all strains produced equivalent expression of all three pro-inflammatory proteins (Fig. 6).

DISCUSSION

The data presented in this study indicate an essential role for intact Rac1 and Cdc42 function in phagocytosis, but not pro-inflammatory protein expression, induced by unopsonized P. aeruginosa. The requirement for Rac1 and Cdc42 in bacterial phagocytosis is consistent with an essential role for actin assembly in the ingestion of this Gram-negative pathogen. In this respect, phagocytosis of Pseudomonas resembles FcγR-mediated phagocytosis and ruffling triggered by colony stimulating factor-1 and the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (11). Precisely how these responses are coupled to activation of Rac1 and Cdc42 is unknown, although they are likely to occur through activation of one or more guanine nucleotide exchange factors (GEFs). Among the best characterized GEFs is Vav, a pleckstrin homology domain containing protein that accelerates GTP exchange by Rac1 and Cdc42 in a tyrosine kinase- and phosphatidylinositol 3-kinase-
Fig. 5. Role of pilin and flagellin in phagocytosis of *P. aeruginosa* by RAW LR/FMLPR.2 cells. A, adherent RAW LR/FMLPR.2 cells were challenged with 4.5 × 10⁵ CFU PAO1 WT (PAO1) or isogenic mutants PA 340 (Pil⁺), AK1152 (Fla⁺), or PA 477 (Pil⁻/Fla⁻) for 45 min at 37 °C. Association indices (number of PAO1 bound per 100 RAW LR/FMLPR.2 cells; *black bars*) and phagocytosis indices (number of PAO1 ingested per 100 RAW LR/FMLPR.2 cells; *hatched bars*) were calculated as described under “Experimental Procedures.” B, phagocytosis expressed as percent of total cell-associated bacteria. Data represent mean ± S.E., *n* = 3.

Fig. 6. Role of *Pseudomonas* pilin and flagellin on expression of COX-2, iNOS, and TNF-α in RAW LR/FMLPR.2 cells. Adherent RAW LR/FMLPR.2 cells (5 × 10⁵) were either not challenged (lane 1) or challenged with 4.5 × 10⁵ (lanes 4, 7, and 10), 4.5 × 10⁴ (lanes 3, 6, and 9), or 4.5 × 10³ (lanes 2, 5, and 8) CFU of PAO1 (lanes 2-4), PA 340 (Pil⁺) (lanes 5-7), or AK1152 (Fla⁺) (lanes 8-10) for 5 h at 37 °C. Cells were subjected to detergent lysis, SDS-polyacrylamide gel electrophoresis, and immunoblotting with the indicated antibodies. Molecular weight markers appear at the left. Similar results were seen in four independent experiments.

dependent manner (32, 33). However, macrophages derived from Vav1 knock-out mice do not demonstrate impaired phagocytosis or membrane ruffling,² and phosphatidylinositol 3-kinase inhibitors do not block FcR-directed actin assembly (34). Another recently characterized Rho family GEF is SopE, a protein derived from *S. typhimurium* that is introduced into epithelial cells by a type III secretion system, thereby stimulating localized actin assembly and phagocytosis (35). *P. aeruginosa* also expresses a type III secretion system (36), although it is unknown whether it plays a role in its phagocytosis by macrophages. Macrophages express multiple phagocytosis-promoting receptors, including several types of Fc receptors, complement receptor 1, complement receptor 3, and the macrophage mannose receptor. All of these have been implicated in phagocytosis of *P. aeruginosa* (21). Since phagocytosis mediated by these receptors can be triggered by inert particles opsonized with their respective ligands, it is doubtful that a type III secretion system is indispensable for *Pseudomonas* phagocytosis. In addition, because a functional type III secretion system requires bacteria-target cell contact (36), our findings using poorly adherent Pil⁻ or Fla⁻ bacterial strains indicate that a type III secretion system also is not required for pro-inflammatory gene expression in murine macrophages.

Pro-inflammatory gene induction expressed by various bacteria has been ascribed to phagocytosis *per se* (1–4, 6, 7). These studies employed cytochalasins, fungal metabolites that inhibit actin assembly. Addition of cytochalasins to cells produces dramatic alterations in the cytoskeleton independent of phagocytosis. Many cellular functions are adversely affected by disruption of cytoskeletal integrity, including protein synthesis (37) and insulin-stimulated DNA synthesis, c-Fos expression, and mitogen-activated protein kinase activation (38). Cytochalasins have been reported either to inhibit (39) or have no effect (40) on NF-κB activation induced by microbial pathogens. We found that cytochalasins inhibited expression of COX-2, iNOS, and TNF-α induced by PAO1 (data not shown), but we could not ascribe this to a specific blockade of phagocytosis. Since inhibition of Rac1 and Cdc42 led to impaired phagocytosis but not pro-inflammatory protein expression, this suggests that bacterial phagocytosis is not required for gene expression. Indeed, results using adhesin-deficient strains of PAO1 (Fig. 6) demonstrate that bacterial ingestion or attachment does not play a major role in the activation of NF-κB or the promotion of NF-κB-dependent gene expression in murine macrophages. *P. aeruginosa* secretes membrane vesicles into the medium (for review, see Ref. 41). These vesicles contain cell wall components, such as lipopolysaccharide (LPS) and other potential inflammatory mediators, including proteases, alkaline phosphatase, phospholipase C, and pro-elastase. It is likely that one or more of these is responsible for induction of iNOS, COX-2, and TNF-α. We found that polymyxin B, which chelates and neutralizes LPS, partially inhibited nuclear translocation of p65 NF-κB induced by highly diluted bacterial supernatants; however, this inhibition was overcome by use of more concentrated supernatants, reflecting either a molar excess of LPS or the presence of additional pro-inflammatory substances (data not shown).

The mechanism of activation of NF-κB is under intense scrutiny (for review, see Ref. 42). This ubiquitous transcription factor is activated by many stimuli, including LPS (43) and a variety of Gram-positive (44, 45) and Gram-negative (40, 46, 47) bacteria. A role for Rho family GTPases in the activation of NF-κB has been reported for several agonists, including interleukin-1 (16) and TNF-α (48). The mechanism by which Rac1 participates in activation of NF-κB may involve the production of reactive oxygen intermediates, such as H₂O₂, by a Rac-sensitive pathway, and oxidation of a kinase or phosphatase that regulates either IκB kinase or another component of the NF-κB signaling pathway. Addition of exogenous H₂O₂ is capable of activating NF-κB in several lymphocyte and fibroblast cell lines (19), and antioxidants inhibited Rac-mediated NF-κB-dependent gene expression (16). However, the activation of NF-κB by TNF-α was not inhibited by expression of Rac1 N17 (48), arguing against a requisite role for Rac-dependent oxidant generation in the activation of NF-κB. In addition, activation of NF-κB in human neutrophils by *Staphylococcus aureus* was

²S. Greenberg and V. L. Tybulewicz, unpublished data.
insensitive to several anti-oxidants (49) and N-acetylcysteine failed to inhibit interleukin-1- and TNF-activated NF-κB in EL4.NOB-1 and KB cells, respectively (50). Together, these data argue that activation of NF-κB does not necessarily depend on oxidant generation. The sensitivity to anti-oxidants of whose overexpression triggers NF-

cytoskeletal changes failed to induce nuclear translocation of alleles of either Rac1 or Cdc42 sufficient to induce marked likely to be cell type- and stimulus-specific.

... data argue that activation of NF-

autocrine/paracrine pathway (26). This implies that Rac is not... these GTPases in NF-

10. McDonald, P. P., and Cassatella, M. A. (1997) Proc. Natl. Acad. Sci. U. S. A. J. Gen. Virol. 75, 849–856
11. Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M., and Greenberg, S. (1997) J. Exp. Med. 186, 1487–1494
12. Massol, P., Montcourrier, P., Guilletmot, J.-C., and Chavrier, P. (1998) EMBO J. 17, 6219–6229
13. Caron, E., and Hall, A. (1998) Science 282, 1717–1721
14. Chen, L.-M., Hobbie, S., and Galan, J. E. (1996) Science 274, 2115–2118
15. Minden, A., Lin, A. N., Claret, F. X., Abo, A., and Karin, M. (1995) Cell 81, 1147–1157
16. Sulciner, D. J., Iriati, K., Xu, S., Ferrans, V. J., Goldschmidt-Clermont, P., and Finkel, T. (1996) Mol. Cell. Biol. 16, 7115–7121
17. Perona, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R., and Lacal, J. C. (1997) Genes Dev. 11, 463–475
18. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G., and Segal, A. W. (1991) Nature 353, 668–670
19. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) EMBO J. 10, 2247–2258
20. Wilson, R., and Dowling, R. B. (1998) Thorax 53, 213–219
21. Speert, D. P., Wright, S. D., Silverstein, S. C., and Mah, B. (1988) J. Clin. Invest. 82, 872–879
22. Rassekh, W. C., Baird, S., Ralph, P., and Nakoinz, I. (1978) Cell 15, 261–267
23. Lamarche, N., Tapon, N., Stowers, L., Barbello, P. D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996) Cell 87, 519–529
24. Tang, H., Kays, M., and Prince, A. (1995) Infect. Immun. 63, 1278–1285
25. Drake, D., and Montie, T. C. (1998) J. Gen. Microbiol. 134, 43–52
26. Tapon, N., Nagata, K., Lamarche, N., and Hall, A. (1998) EMBO J. 17, 1395–1404
27. Allen, W. E., Jones, G. E., Pollard, J. W., and Ridley, A. J. (1997) J. Cell Sci. 110, 767–770
28. Sweet, M. J., and Hume, D. A. (1996) J. Leukocyte Biol. 60, 8–26
29. Tebo, J. M., Chaquen, W., O’Connor, T., and Hall, A. T. (1994) J. Immunol. 153, 4713–4720
30. Wrighton, C. J., Hofer-Warbinek, R., Moll, T., Kytyn, R., Bach, F. H., and de Martin, R. (1996) J. Exp. Med. 183, 1013–1022
31. Mahenthiralingam, E., and Speert, D. P. (1995) Infect. Immun. 63, 4519–4523
32. Crespo, P., Schuesel, K. E., Ostrom, A. A., Gutkind, J. S., and Bustelo, X. R. (1997) Nature 385, 159–172
33. Han, J. W., Luby-Phelps, K., Das, B. S., Shu, X. D., Xia, Y., Mosteller, R. D., Krishnan, U. M., Falck, J. R., White, M. A., and Broek, D. (1998) Science 279, 558–560
34. Cox, D., Tseng, C.-C., Bjeckic, G., and Greenberg, S. (1999) J. Biol. Chem. 274, 1240–1247
35. Hoft, W. D., Chen, L.-M., Schuebel, K. E., Custe, S. R., and Galan, J. E. (1998) Cell 93, 815–826
36. Frank, D. W. (1997) Mol. Microbiol. 26, 621–629
37. Fasahauer, M., Iwrg, M., and Glasser, D. (1998) Eur. J. Cell Biol. 77, 188–195
38. Tsuchida, T., Bergman, A., Somwar, R., Taha, C., Aksenov, M., Cruz, T. F., Kipl, A., and Downey, G. P. (1998) J. Biol. Chem. 273, 28322–28331
39. Sporn, L. A., Sahn, S. K., Lerner, N. B., Marder, V. J., Silverman, D. J., Turpin, L. C., and Schwach, A. L. (1997) Infect. Immun. 65, 2786–2791
40. Hoft, W. D., Goebel, W., Serling, K., and Kuhn, M. (1994) Infect. Immun. 62, 2740–2747
41. Kadurugamuwa, J. L., and Beveridge, T. J. (1997) J. Antimicrobial. Chemother. 40, 615–621
42. Merchuro, F., and Manning, A. M. (1999) Curr. Opin. Cell Biol. 11, 226–232
43. Sen, R., and Baltimore, D. (1986) Cell 47, 921–928
44. Busam, K., Gieringer, C., Fire, C., and Mohrland, H. P. (1992) Infect. Immun. 60, 2008–2015
45. Hoft, W. D., Goebel, W., Fiedler, P., Sokolovic, Z., and Kuhn, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8394–8399
46. Noel, R. J., Sato, T. T., Mendez, C., Johnson, M. C., and Pohlman, T. H. (1995) Infect. Immun. 63, 4046–4053
47. Li, J. D., Feng, W. J., Gallup, M., Kim, J. H., Gum, J., Kim, Y., and Basbaum, C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5718–5723
48. Montaner, S., Perona, R., Saniger, L., and Laci, J. C. (1998) J. Biol. Chem. 273, 12779–12785
49. Vilela, M., Hampton, M. B., and Winterbourn, C. C. (1998) FEMS Lett. 432, 40–44
50. Brennan, P., and O’Neill, L. A. (1995) Biochim. Biophys. Acta 1260, 167–175