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Cross-Talk between CD14 and Complement Receptor 3 Promotes Phagocytosis of Mycobacteria: Regulation by Phosphatidylinositol 3-Kinase and Cytohesin-1[1]

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The glycosylphosphatidyl anchored molecule CD14 to the monocyte membrane plays a prominent role in innate immunity, and the paradigms for CD14 selective signaling are beginning to be elucidated. In this study, transfected human monocytic cell line THP-1 and Chinese hamster ovary (CHO) fibroblastic cells were used to examine phagocytosis of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Flow cytometry was combined with molecular and biochemical approaches to demonstrate a dual mechanism for BCG internalization involving either CD14 alone or a CD14-regulated complement receptor (CR)3-dependent pathway. Phagocytosis by CD14-positive THP-1 cells was attenuated by phosphatidylinositol-3 inhibitors LY294002 and wortmannin and experiments using transfected CHO cells showed substantial accumulation of phosphatidylinositol-3,4,5-trisphosphate at the BCG attachment site in CHO cells expressing CD14 and TLR2 suggesting that bacteria bind to CD14 and use TLR2 to initiate a PI3K signaling pathway. Additional experiments using blocking Abs showed that anti-TLR2 Abs inhibit phagocytosis of BCG by THP-1 cells. Furthermore, knockdown of cytohesin-1, a PI3K-regulated adaptor molecule for β_2 integrin activation, specifically abrogated CD14-regulated CR3 ingestion of BCG consistent with the observation of physical association between CR3 and cytohesin-1 in cells stimulated with mycobacterial surface components. These findings reveal that mycobacteria promote their uptake through a process of "inside-out" signaling involving CD14, TLR2, PI3K, and cytohesin-1. This converts low avidity CR3 into an active receptor leading to increased bacterial internalization. ***The Journal of Immunology,*** **2005, 174: 4210–4219.**

Several receptors involved in the internalization of pathogenic mycobacteria by macrophages have been tentatively characterized but not extensively studied. Binding of mycobacteria to macrophages is believed to occur in cholesterol-rich domains of the host cell plasma membrane (1) and involvement of β_2 integrins (complement receptor (CR)[3] 3 and CR4), together with other molecules including the mannose receptor, CR1 and CD14 (2) has been suggested. Other surface molecules, such as TLRs, are also involved in mycobacterial interactions with phagocytic cells (3).

In monocytes and immature macrophages, which do not express the mannose receptor (4), CR3 and CD14 have been shown to be potential gates for entry of mycobacteria (5, 6). However, whether these two receptors act individually or cooperatively is not known. Indeed, the avidity of CR3 for its ligands in resting cells is low and CR3 must be activated to mediate stable binding (7–9). Interest in signaling events that link cell stimulation to the activation of β_2 integrins has been considerable in recent years (reviewed in Refs. 10 and 11). Pioneering studies by Kolanus et al. (12) led to the discovery of an adaptor protein cytohesin-1, which interacts with the cytoplasmic tail of CD18 leading to changes in the functional properties of β_2 integrins. Cytohesin-1 contains a pleckstrin homology domain that binds the PI3K metabolite, phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-P_3), leading to changes in properties of the protein (13, 14). These findings have suggested a role for PI3K in regulating the activity of β_2 integrins (15, 16), which possibly includes CR3.

The mycobacterial cell surface glycolipid lipoarabinomannan (LAM) is known to enhance phagocytosis of mycobacteria (17). It has also been shown that LAM-regulated phagocytosis is dependent at least in part on CR3 (18). Superficially, these findings appear somewhat paradoxical because the dominant signaling pathway for LAM that has been described involves the membrane receptors CD14 and TLR2 and not CR3 (19–21). Whether CD14, TLR2, and CR3 cooperate to bring about optimal uptake of mycobacteria and if so how they may be linked are important unanswered questions. In the present study, we demonstrated that binding of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) to CD14 induces an inside-out signaling pathway, which involves TLR2, PI3K, and cytohesin-1 leading ultimately to enhanced CR3-dependent bacterial internalization.

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[3] Abbreviations used in this paper: CR, complement receptor; PtdIns, phosphatidylinositol; BCG, bacillus Calmette-Guérin; PPD, purified protein derivative; CHO, Chinese hamster ovary; S-oligo, phosphorothioate-modified oligonucleotide; RITC, rhodamine isothiocyanate; MFI, mean fluorescence intensity; LAM, lipoarabinomannan; LBP, LPS binding protein.

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Materials and Methods

Reagents and chemicals

Endotoxin-free RPMI 1640, HBSS, penicillin/streptomycin, 1 M HEPES solution, and nonessential amino acids were from StemCell Technologies. Latex beads (4 μm diameter) were from Interfacial Dynamics. Wortmannin, trypsin-EDTA, L-α-phosphatidylinositol, protease inhibitor mixture, and PMSF were obtained from Sigma-Aldrich. LY294002 and G418 were from Calbiochem. Human AB^+ serum was selected from purified protein derivative $(PPD)^-$ volunteer donors and tested for the absence of IgG directed to mycobacteria cell surface. Purified human LPS binding protein (LBP) was from HyCult Biotechnology. Protein A-agarose and electrophoresis reagents were purchased from Bio-Rad.

Antibodies

Polyclonal rabbit Ab to the BCG membrane were a gift from Dr. R. Stokes (University of British Columbia, Vancouver, British Columbia, Canada). Anti-human CR3 mAbs (clone LM2 and M1/70) were from Hybridoma Bank (University of Iowa, Iowa City, IA) and FITC-labeled anti-CR3 (clone VIM12) was from Caltag Laboratories. Anti-CD14 mAb, clone 3C10, was a gift from Dr. W. C. Van Voorhis (University of Washington, Seattle, WA) and 63D3 was a gift from Dr. P. S. Tobia (The Scripps Research Institute, La Jolla, CA). Anti-cytohesin-1 mAb (clone 7H2) was a gift from Dr. W. Kolanus (Ludwig-Maximilians-Universität, München, Germany) and rabbit polyclonal anti-cytohesin-1 (O139) was previously described (22). Anti-TLR2 (clone TL2.1), anti-TLR4 (clone HTA125), and FITC-labeled anti-CR3 activation epitope (clone CBRM1/5) were from eBiosciences. Anti-PI3K mAb (clone UB93-3) was from Upstate Biotechnology and anti-PtdIns-3,4,5-P_3 (clone RC6F8, IgM) was from Molecular Probes. Irrelevant isotype-matched mAbs were from Caltag Laboratories and fluorescent secondary Abs were obtained from Sigma-Aldrich.

Cell lines

The monocytic cell lines THP-1wt (THP-1 cells stably expressing glycosylphosphatidylinositol-anchored CD14) and THP-1rsv (THP-1 cells transfected with vector alone) were kindly provided by Dr. R. Ulevitch (The Scripps Research Institute). THP-1 cells were cultured in RPMI 1640 supplemented with 5% FCS (Invitrogen Life Technologies), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 1 mg/ml G418. Chinese hamster ovary (CHO) fibroblastic cells CHO/CR3, CHO/CD14, CHO/CD14/TLR2, and CHO expressing vector alone (CHO/rsv) were obtained from Dr. D. T. Golenbock (Boston Medical School, Boston, MA) and cultured in DMEM supplemented with 5% FCS, L-glutamine, 1% nonessential amino acid mixture, penicillin/streptomycin, 10 mM HEPES, and 1 mg/ml G418.

Bacterial strains

Cultures of *M. bovis* BCG and *Escherichia coli* expressing GFP were provided by Dr. Y. Av-Gay (University of British Columbia, Vancouver, British Columbia, Canada). To prepare red-fluorescent mycobacteria, 10^9 organisms from the parental BCG strain were suspended in 1 ml of 7H9 broth and labeled by incubation with rhodamine isothiocyanate (RITC; Sigma-Aldrich) at 1 μg/ml for 2 h at 37°C. Thereafter, RITC-labeled bacteria were washed twice with 7H9 and stored at −70°C.

Mycobacterial preparations

Cell wall fractions and LAM from *Mycobacterium tuberculosis* (H_{37}Rv strain) were provided by Dr. J. Belisles (Colorado State University, Fort Collins, CO) through a TB Research Materials and Vaccine Testing contract (National Institutes of Health, National Institute of Allergy and Infectious Diseases NO1-A1-75320). Endotoxin contamination in all these preparations was below 1 ng/mg as determined by the *Limulus* amebocyte lysate assay.

Phagocytosis assay

Thawed *M. bovis* BCG samples were pelleted and suspended in 1 ml of RPMI 1640 and clumps were disrupted by multiple passages through a 25-gauge needle. Bacteria were opsonized in RPMI 1640 plus 10% AB^+ and PPD^- serum and washed twice with RPMI 1640. Cells (10^6) were exposed to opsonized BCG at a 20:1 ratio in 1 ml of serum in six-well plates. THP-1 cells were incubated for 2 h and CHO cells for 6 h. Partially attached, noningested bacteria were removed by 10 min treatment with trypsin-EDTA at 37°C and extensive washing with HBSS as described (1). The proportion of cells loaded with bacteria was measured by using a FACSCalibur flow cytometer (BD Biosciences). Relative fluorescence intensity of 10,000 cells was recorded as single-parameter histograms (log scale, 1024 channels, 4 decades).

In vitro PI3K assay

Cell lysates for analysis of PI3K were prepared in 20 mM Tris, pH 8.0, 1% Triton X-100, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 1 mM PMSF, and protease inhibitor mixture. Aliquots of lysates adjusted for protein concentration (500 μg of protein) were incubated for 3 h at 4°C with UB93-3 mAb (anti-PI3K), and immune complexes were adsorbed onto protein A-agarose for 30 min. The complexes were washed with 10 mM Tris-HCl, pH 7.4 and assayed for lipid kinase activity as previously described (16, 23). To ensure that PI3K levels remained equivalent at the end of the immunoprecipitation process, 10% from each treatment sample were collected during the last wash in separate tube and analyzed by SDS-PAGE and immunoblotting with Abs to the p85 PI3K subunit as described (24).

Fluorescence microscopy

Intracellular staining was performed on adherent cells to tissue culture-treated coverslips (Fisher Scientific) in 24-well. Cells were washed with PBS and fixed for 15 min at 37°C with 2.5% paraformaldehyde/PBS, then washed three times and permeabilized in PBS containing 0.2% saponin and 10% normal goat serum for 5 min. Cells were then incubated with primary mAb within PBS/saponin/normal serum for 30 min at room temperature, washed, and stained with FITC-conjugated (or Texas Red) secondary Ab. After final wash, coverslips were mounted on slides and examined for two-color signals using an epifluorescence microscope (Zeiss Axioplan II; Carl Zeiss) as described (25, 26).

Sense and antisense oligonucleotides

Cytohesin-1 expression in THP-1wt cells was inhibited by antisense strategy using phosphorothioate-modified oligonucleotides (S-oligos) as previously described (16). S-oligos were made in both sense and antisense orientations as follows: sense, 5′-ATG GAG GAG GAC GAC AGC TAC-3′; antisense, 5′-GTA GCT GTC GTC CTC CTC CAT-3′. THP-1wt cells (10^6) were incubated for 2 h at 37°C and 5% CO_2 in 250 μl of RPMI 1640 containing 2.5% LipofectAMINE (Invitrogen Life Technologies) and 5 μM S-oligos. After incubation, the medium was adjusted to 1 ml and supplemented with 10% FCS, and cells were cultured for an additional 18 h.

Flow cytometry analysis of "pull down" cytohesin-1

Latex beads (4 μm; Interfacial Dynamics) were coated with purified rabbit anti-cytohesin IgG in 25 mM MES buffer (pH 5.5) and blocked with BSA according to manufacturer instructions. Control and stimulated cells were lysed in buffer containing 100 mM Tris, pH 7.5, 150 mM NaCl, 1% Brij 58 (Pierce), 1 mM PMSF, and protease inhibitor mixture. Insoluble material was removed by centrifugation and the supernatant was incubated with coated beads for 30 min at 4°C. Beads were then washed and stained with FITC-labeled anti-CR3 or anti-PtdIns-3,4,5-P_3 or irrelevant mAbs for 30 min at 4°C. Latex beads were then washed and analyzed by flow cytometry using specific side scatter and forward scatter parameters that identify latex beads as previously described (26). FL1 (green fluorescence) signals, which correspond to the binding of fluorescent Ab, were recorded and mean fluorescence intensity (MFI) was determined for individual fluorescence histograms.

Statistical analysis

All data are expressed as the mean ± SD. Statistical analysis was performed using Student's t test. Values of $p < 0.05$ were considered to be significant.

Results

Expression of CD14 confers a phagocytic phenotype for BCG that is partially dependent upon CR3

To examine whether CD14 participates in the phagocytosis of mycobacterium, we used THP-1 cells stably transfected with either CD14 (THP-1wt) or vector alone (THP-1rsv). Both THP-1wt and THP-1rsv cell lines express significant and similar levels of CR3, whereas only THP-1wt expresses CD14 (Table I). THP-1wt cells were exposed to BCG expressing GFP for 2 h and treated with trypsin-EDTA (to detach noningested bacteria) and phagocytosis was quantified by flow cytometry. Control experiments combining

Table I. *Expression of surface receptors on THP-1 and CHO cells*[a]

Cell Lines	MFI Indices[b]		
	LM2	3C10	TL2.1
THP-1rsv	12.7	2.1	17.0
THP-1wt	11.6	74.9	16.3
CHO/CR3	12.6	1.4	1.3
CHO/CD14	1.1	61.7	1.2
CHO/TLR2	1.2	1.5	14.8
CHO/CD14/TLR2	1.2	63.2	15.1

[a] Cells were stained for 20 min at 4°C with anti-CR3 mAb (clone LM2) or anti-CD14 (clone 3C10) or anti-TLR2 (clone TLR2.1). Cells were also incubated with irrelevant isotype-matched IgG to control for nonspecific binding. Samples were then washed, stained with FITC-conjugated goat anti-mouse IgG, and analyzed by flow cytometry.

[b] MFI indices correspond to the ratio of MFI of cells incubated with specific Ab to MFI of cells stained with irrelevant isotype-matched IgG.

flow cytometry and confocal microscopy indicated that the fluorescent signal detected by flow cytometry corresponded to internalized bacilli rather than to GFP-BCG adherent to the cell surface (Fig. 1). Phagocytosis was negligible in absence of serum (data not shown).

THP-1rsv ($CD14^-$) cells were only marginally phagocytic (~10% positive) and transfection with CD14 (THP-1wt) markedly enhanced phagocytosis of BCG (>60%) (Fig. 2*A*). Replacement of normal serum with LBP reduced phagocytosis by THP-1wt cells to

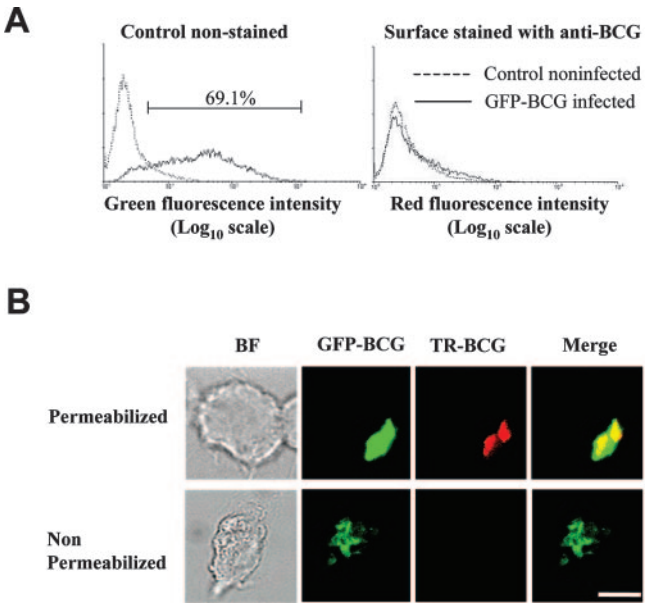


FIGURE 1. Quantitative detection of internalized GFP-BCG by flow cytometry. *A*, THP-1wt cells were incubated or not (control) for 2 h at 37°C with serum-opsonized GFP-BCG. Surface attached bacteria were removed by trypsinization and HBSS washing. Samples were then fixed and analyzed by flow cytometry. Results are expressed as green fluorescence histogram of cells ingesting GFP-BCG (*left*) or red fluorescence histogram of cells ingesting bacteria and surface stained with specific rabbit IgG anti-BCG membrane (*right*) and revealed by PE-conjugated goat anti-rabbit IgG. Percentage (*left*) indicates the proportion of cells containing BCG as calculated by WinMDI 2.8 software. *B*, After trypsinization, fractions of cells were plated on coverslips, fixed, permeabilized or not, and stained with specific rabbit IgG anti-BCG membrane. Bound Ab was detected with Texas Red-conjugated goat anti-rabbit IgG. Samples were then analyzed with digital confocal microscopy for green and red fluorescence. GFP signal (green) and anti-BCG IgG (red) are displayed and yellow signals indicate binding of rabbit IgG to the BCG membrane. Bar represents 10 μm.

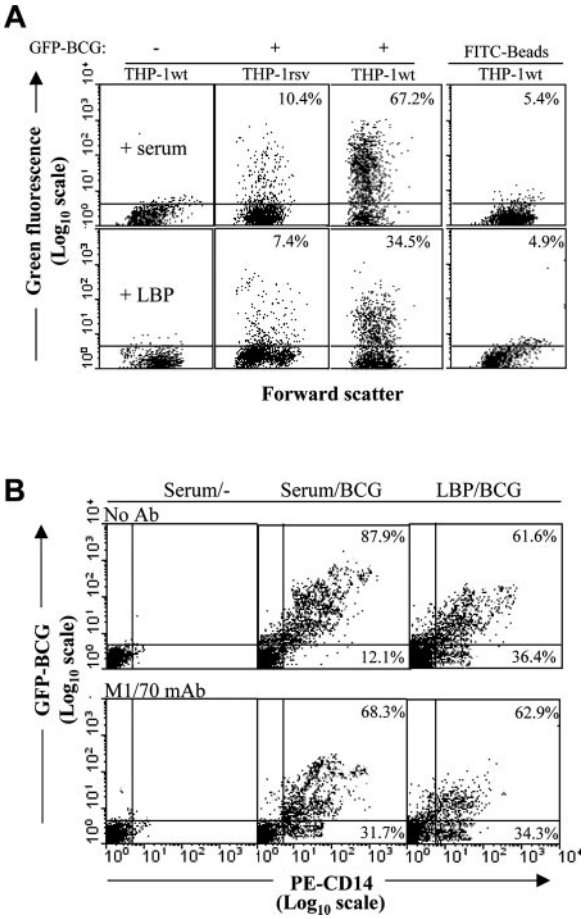


FIGURE 2. CD14- and CR3-dependent phagocytosis of BCG. THP-1rsv or THP-1wt (*A*) or mononuclear cells (*B*), isolated from normal human blood by centrifugation over Histopaque as previously described (66), were incubated in presence of GFP-BCG or FITC latex beads, opsonized with either 10% PPD^- serum or 200 ng/ml LBP (bacteria or beads to cell ratio of 20:1). Surface attached bacteria or beads were removed by trypsin treatment and mononuclear cells were submitted to additional staining with anti-CD14 mAb (clone LM2) and PE-conjugated goat anti-mouse IgG. The percentage of cells ingesting BCG or beads was measured by flow cytometry. Results are expressed as dot blots combining either forward scatter/green fluorescence (THP-1 cells) or red/green fluorescence (mononuclear cells) parameters. The signals shown corresponding to cells that were not incubated with bacteria represent levels of autofluorescence and were used as negative controls to set gates to include only THP-1 cells and CD14-positive mononuclear cells that had ingested fluorescent bacteria. The percentage shown indicates the proportion of cells containing BCG or beads as calculated by WinMDI 2.8 software.

~35% suggesting that there are opsonins in serum other than LBP, perhaps C3bi or others required for optimal uptake of BCG by THP-1wt cells. Exposure of THP-1wt cells to fluorescent inert latex beads led to minimal uptake indicating that expression of CD14 does not generically enhances phagocytic efficiency. As was the case for THP-1wt cells, primary human CD14-positive monocytes were also able to ingest BCG organisms in the presence of serum and LBP. Furthermore, LBP-dependent phagocytosis was maintained in the presence of blocking anti-CR3 mAb (Fig. 2*B*).

The role of CD14 in phagocytosis was investigated further by competitive inhibition with neutralizing mAbs. As shown in Fig. 3*A*, preincubation of THP-1wt cells with anti-CD14 mAb, 3C10, before exposure to BCG significantly reduced phagocytosis (~65% inhibition). The contribution of CR3 in this model was also examined using two different anti-CR3 mAb, M1/70 mAb, which

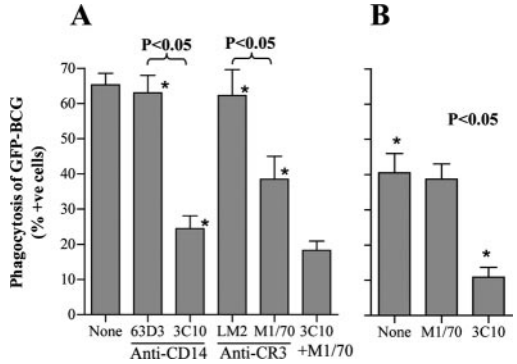


FIGURE 3. Competitive inhibition of phagocytosis by mAb to CR3 and CD14. THP-1wt cells were incubated for 30 min at room temperature with mAbs to either CD14 (clone 3C10 or 63D3) or CR3 (clone M1/70 or LM2) at a final concentration of 20 μg/ml. Cells were then assayed for phagocytosis of GFP-BCG opsonized with either 10% serum (*A*) or 200 ng/ml LBP (*B*). The proportions of cells ingesting BCG were determined as described in Fig. 2. Values of phagocytosis percentage in control cells were significantly different from those measured in cells pretreated with blocking mAbs 3C10 and M1/70; $p < 0.05$. The data shown are the mean ± SD of three independent experiments.

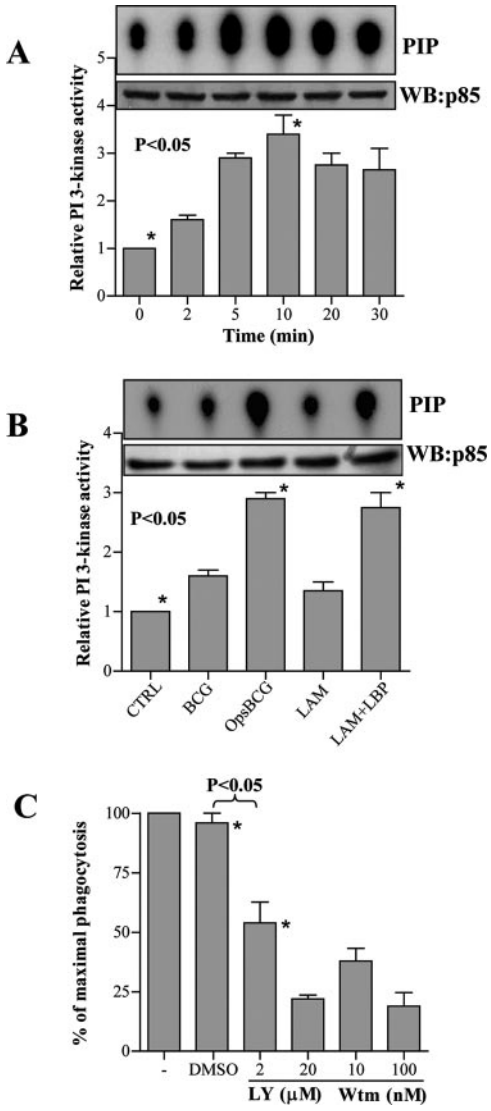


FIGURE 4. Phagocytosis of *M. bovis* BCG is dependent on PI3K. *A*, THP-1wt cells (5×10^6) were exposed to serum-opsonized BCG (bacteria to cell ratio of 20:1) in 5 ml of RPMI 1640 in 10 cm diameter culture dishes for the indicated time at 37°C. At the end of each time point culture dishes were immediately transferred to 4°C. *B*, Cells were prepared as in *A* and stimulated for 10 min with BCG alone or serum-opsonized BCG, LAM (5 μg/ml) alone, or LAM in presence of 200 ng/ml LBP. Cell samples (*A* and *B*) were assayed for PI3K activity as previously described (22, 23). Spots corresponding to phosphatidylinositol phosphate (PIP) were cut and analyzed by scintillation counting. Activities are expressed as fold increase with reference to untreated cells (CTRL). To ensure that PI3K levels were equivalent at the end of the immunoprecipitation, 10% from each treatment sample was collected during the last wash in a separate tube and analyzed by SDS-PAGE and immunoblotting with Abs to the p85 PI3K subunit as previously described (24). *C*, Serum starved THP-1wt cells were incubated for 20 min at 37°C with various concentrations of LY294002 or wortmannin. Serum-opsonized bacteria were then added and cells were incubated for an additional 2 h at 37°C. The proportion of cells ingesting BCG was measured as described in Fig. 2 and the data are presented as a percentage of maximal phagocytosis. The values shown (*A* and *B*) are the mean ± SD of three independent experiments. Values in *C* represent average of three independent experiments.

recognizes an I domain epitope that include the C3bi binding site (27), and LM2/1 mAb, which also binds to the I domain but does not inhibit C3bi binding (28). Pretreatment with M1/70 inhibited phagocytosis of serum-opsonized BCG (40% decrease), whereas LM2/1 mAb had no effect. Phagocytosis was decreased further when anti-CD14 and anti-CR3 were used in combination (~75% inhibition). However, in the presence of LBP, the uptake of BCG was maintained in the presence of anti-CR3 mAb, whereas anti-CD14 brought about a reduction of ~75% (Fig. 3*B*). Specificity of inhibition by anti-CD14 and anti-CR3 mAbs was validated using mAb anti-CD14 clone 63D3 and mAb anti-CR3 clone LM2, which are nonblocking mAbs. Taken together, these data suggest that both CR3 and CD14 are involved in the uptake of BCG and that under serum-free conditions, CD14 is capable of mediating binding of LBP-opsonized organisms, which subsequently triggers events leading to phagocytosis.

CD14-dependent phagocytosis of M. bovis *BCG involves PI3K*

In light of the important role played by PI3K in regulating a variety of cell functions, including phagocytosis (29, 30), the possibility that PI3K regulates CD14-dependent phagocytosis of BCG was examined. As shown in Fig. 4*A*, incubation of THP-1 cells with serum-opsonized BCG brought about a rapid and significant increase in PI3K activity with a maximum response observed within 10 min (~3.5-fold increase). PI3K activity was also significantly increased in cells incubated with LAM in the presence of LBP (3-fold increase) (Fig. 4*B*). PI3K inhibitors were used to examine further the potential involvement of PI3K in CD14-induced phagocytosis. Preincubation with LY294002 inhibited phagocytosis in a dose-dependent manner (Fig. 4*C*) (maximum inhibition 75%) with an IC_{50} ~2 μM, and wortmannin, an inhibitor of PI3K that acts via a distinct mechanism, also attenuated phagocytosis (maximum inhibition 78%) with an IC_{50} below 10 nM. Taken together, these findings suggest that PI3K activation plays a central role in CD14-dependent phagocytosis of BCG.

Mycobacterial LAM up-regulates the avidity of CR3

Stimulation of phagocytic leukocytes adhesion is associated with increased binding of mAb CBRM1/5 that recognizes an "activated" neoepitope on CD11b, the α-chain of CR3. Expression of this neoepitope is considered to reflect increased avidity of CR3 for ligand (28, 31, 32). Therefore, we examined whether the interaction of cell surface CD14 with ligands on the bacterial surface

such as LAM leads to a change in the avidity of CR3. Immunostaining and flow cytometry analysis showed that treatment of THP-1wt cells with either a soluble mycobacterial cell wall fraction or purified LAM induced, respectively, 2-fold and 2.6-fold increases in CBRM1/5 binding (Fig. 5*A*). As a positive control, cells were incubated with the potent CR3 activator PMA (33), which induced a 4.3-fold increase in CBRM1/5 binding. To examine whether activation of CR3 by LAM was regulated by CD14, PI3K or both, THP-1wt cells were preincubated for 20 min with either 3C10 mAb or LY294002 before addition of LAM. Surface staining with CBRM1/5 mAb and flow cytometry analysis showed that LAM-induced changes in CR3 avidity were attenuated by both treatments (56 and 54% inhibition, respectively) (Fig. 5*B*). Taken together, these data suggest that both LAM and other mycobacterial cell wall fraction components of BCG have the capacity to enhance bacterial uptake based upon changes in the avidity of CR3. This process appears to be triggered through cell surface CD14 and is PI3K-dependent.

CD14-dependent phagocytosis involves TLR 2

CD14 inserted into cell membrane through a glycosylphosphatidyl anchor requires proteins of the TLR family to initiate signaling cascades that regulate diverse cellular responses to microbial pathogens (3, 34). CHO cells transfected with surface receptors (Table I) were used to investigate the potential role of TLR2 in regulating CD14-dependent phagocytosis. As shown in Fig. 6*A*, exposure of cells transfected with CD14 or TLR2 alone to opsonized BCG led to a modest degree of bacterial ingestion (10.9 and 13.6%, respectively) and when TLR2 and CD14 were coexpressed

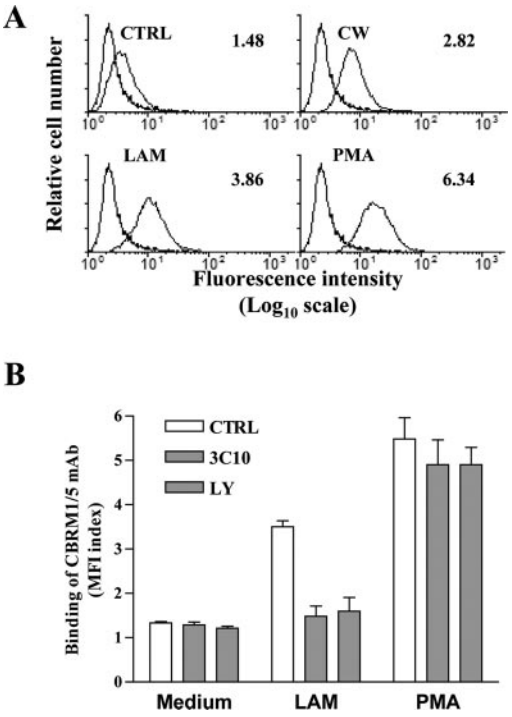


FIGURE 5. Mycobacterial LAM induces the expression of a CR3 activation epitope. *A*, THP-1wt cells were incubated, in the presence of 200 ng/ml LBP, with either a soluble cell wall fraction (50 μg/ml), purified LAM (5 μg/ml), or PMA (100 ng/ml) for 20 min at 37°C. Cells were then stained with FITC-conjugated anti-CR3 mAb (clone CBRM1/5) directed at an activation epitope or irrelevant isotype-matched mAb. *B*, Cells were first incubated for 20 min with either mAb to CD14 (clone 3C10) or LY294002 before exposure to LAM and cells were stained with CBRM1/5 mAb. Samples were then washed, fixed in 2% paraformaldehyde and analyzed by flow cytometry. Fluorescence histograms in *A* represent one of three independent experiments that yielded similar results. Histograms on the *left* correspond to cells stained with irrelevant mAb and the histogram shifted to the *right* corresponds to cells stained with specific mAb. Values in the *top right* indicate MFI, which correspond to the ratio MFI of cells incubated with specific Ab to MFI of cells stained with irrelevant isotype-matched IgG. The data shown in *C* are the mean ± SD of three independent experiments.

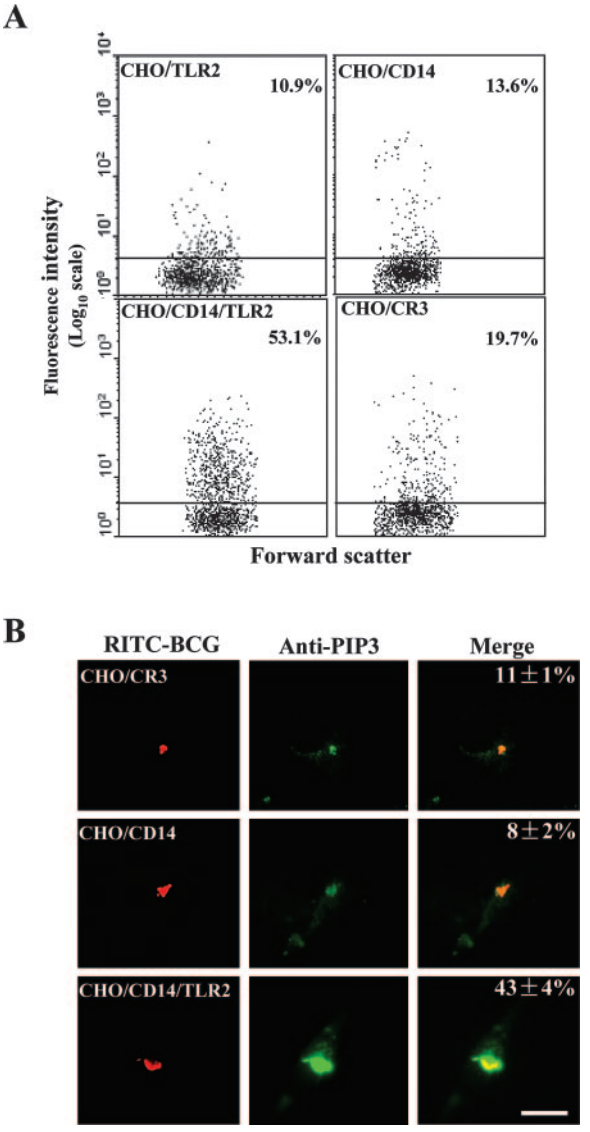


FIGURE 6. CD14-dependent phagocytosis is mediated by TLR2. *A*, CHO cells transfected with TLR2 (CHO/TLR2), CD14 (CHO/CD14), CR3 (CHO/CR3), or CD14 plus TLR2 (CHO/CD14/TLR2) were adhered to six-well culture plates and incubated with serum-opsonized GFP-BCG for 6 h at 37°C. Surface attached bacteria were removed by trypsinization and washing. The proportions of cells ingesting BCG were determined by flow cytometry as described in Fig. 2. Percentages shown indicate average of the proportion of cells ingesting BCG from two independent experiments. Bacterial ingestion by CHO cells transfected with vector alone was below 5%. *B*, Adherent CHO cells to tissue-culture coverslips were exposed to RITC-labeled BCG for 20 min. Cells were then extensively washed, fixed, permeabilized, and stained with anti-PtdIns-3,4,5-P_3 mAb (clone RC6F8, IgM) and FITC-labeled goat anti-mouse IgM. Labeled cells were analyzed with digital confocal microscopy for green and red fluorescence. Images are displayed with red (bacteria) and green (PtdIns-3,4,5-P_3) and yellow signals, the latter indicating colocalization of green and red. Values (*right column*) correspond to the average of positive colocalization of 75 cells from two separate experiments. Bar represents 10 μm.

(CHO/CD14/TLR2), a 4- to 5-fold increase in the proportion of cells ingesting bacteria was observed. Expression of CR3 alone also supported a modest degree of phagocytosis and this was likely due to CR3 activation in response to adherence to plastic. To investigate whether PI3K is involved in TLR2-dependent ingestion of mycobacteria, we examined the intracellular distribution of the PI3K product PtdIns-3,4,5-P_3 in vivo. Cells were exposed to RITC-BCG for 20 min and extensively washed to remove unbound bacteria. Cells were then permeabilized and stained with mAb RC6F8 followed by FITC-conjugated secondary Ab. The RC6F8 mAb recognized specifically PtdIns-3,4,5-P_3, with minimal cross-reactivity with PtdIns-(4,5)-diphosphate and for the two monophosphates PtdIns-(3)-monophosphate and PtdIns-(4)-monophosphate (35). The results in Fig. 6*B* show a marked colocalization of PtdIns-3,4,5-P_3 with RITC-BCG in CHO/CD14/TLR2, but not in CHO/CR3 or CHO/CD14. The role of TLR in phagocytosis was also investigated in THP-1 cells and the data in Table II show that phagocytosis of BCG, or LAM-coated latex beads, by THP-1wt cells was inhibited (~40 and ~45%, respectively) by blocking anti-TLR2, whereas anti-TLR4 mAb was without effect. Conversely, and as expected, anti-TLR2 mAb had a minimal effect on phagocytosis of either *E. coli* or LPS-coated beads, which was more efficiently blocked with anti-TLR4 mAb (~43 and ~60%, respectively). These findings suggest that after binding of bacteria to CD14, TLR2 is required to bring about activation of PI3K and the subsequent accumulation of PtdIns-3,4,5-P_3 in the phagocytic cup and bacterial internalization.

CR3-mediated phagocytosis of M. bovis *BCG is regulated by cytohesin-1*

Cytohesin-1 is an adaptor protein that has been shown to bind specifically to the cytoplasmic domain of CD18 and to regulate adhesiveness of the leukocyte integrin LFA-1 (CD11a/CD18) (12). The interaction of cytohesin-1 with LFA-1 is inducible and is regulated by the PI3K product PtdIns-3,4,5-P_3 that binds to the cytohesin-1 pleckstrin homology domain (15, 36). These findings suggested the hypothesis that CD14/PI3K dependent enhancement of CR3 avidity brought about by mycobacterial cell wall fraction components (Fig. 5) may involve cytohesin-1. To investigate this possibility, cytohesin-1 expression was inhibited by incubation of THP-1wt cells in the presence of antisense S-oligos spanning the cytohesin-1 as previously described (16). As shown in Fig. 7*A*, treatment of cells with antisense S-oligo to cytohesin-1 mRNA, but not with control sense S-oligo, eliminated the expression of this protein concomitant with significant attenuation of serum-dependent uptake of BCG (53% inhibition) (Fig. 7*B*). In contrast, anti-

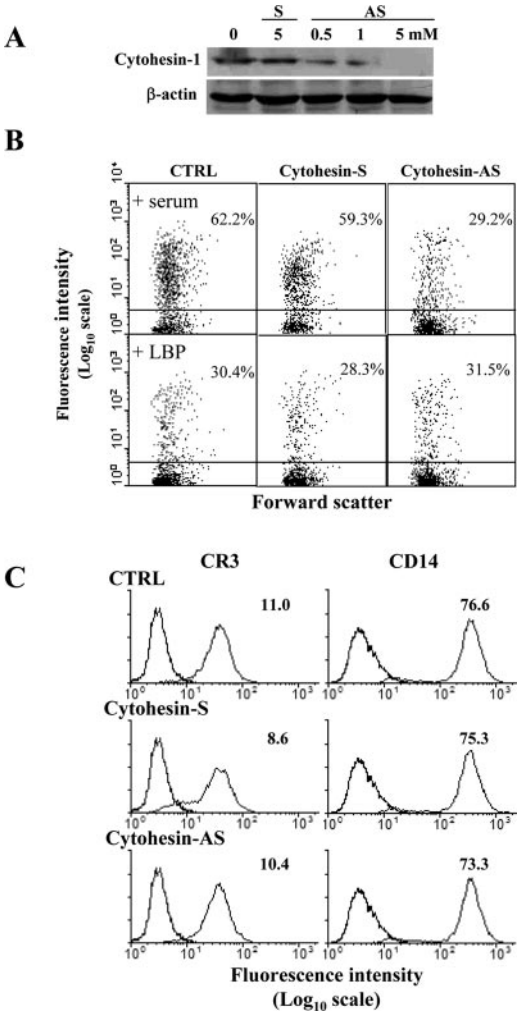


FIGURE 7. Antisense S-oligos to cytohesin-1 attenuates CR3-mediated phagocytosis of *M. bovis* BCG. THP-1wt cells were incubated with sense (S) or antisense (AS) S-oligos to cytohesin-1 messenger for 18 h. *A*, Whole cell lysates were separated by SDS-PAGE, Western blotted, and immunodetected with rabbit polyclonal anti-cytohesin-1. *B*, Cells treated with 5 μM S-oligos were washed and examined in the phagocytosis assay as described in Fig. 2. Percentages indicate proportion of cells ingesting BCG. *C*, Control and S-oligo-treated cells were stained with anti-CR3 (clone LM2) or anti-CD14 (clone 3C10) mAb or irrelevant isotype-matched IgG and analyzed by flow cytometry as described in Fig. 5. Values indicate MFI. Data shown represent one of three independent experiments with similar results.

Table II. *Blocking anti-TLR2 mAb attenuates phagocytosis of BCG by THP-1 cells*[a]

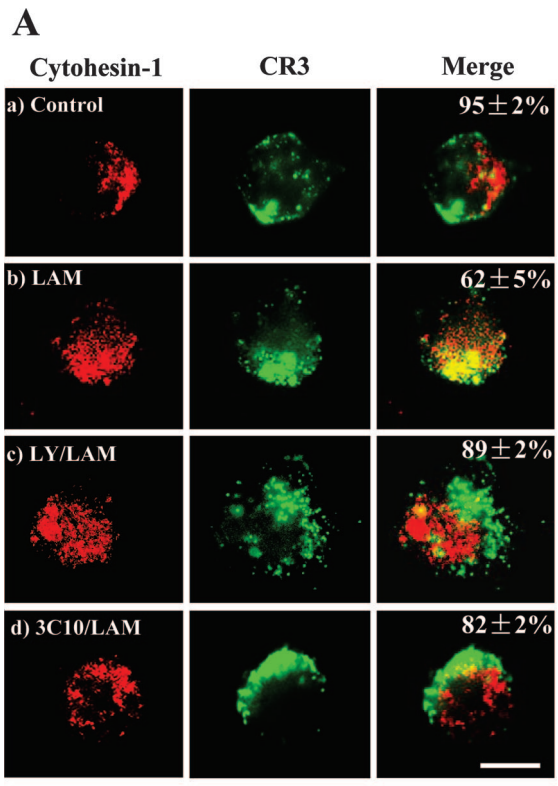
mAb	Percentage of Phagocytosis ± SD			
	GFP-BCG	GFP-*E. coli*	F-LTX-LAM	F-LTX-LPS
None	62.2 ± 4.1	67.7 ± 3.7	16.8 ± 2.1	18.1 ± 2.9
Control IgG2a	56.0 ± 4.6	65.3 ± 4.0	15.6 ± 3.1	15.8 ± 3.2
TL2.1	36.3 ± 3.8*	51.9 ± 3.7	7.5 ± 2.1*	15.8 ± 1.9
HTA125	52.4 ± 3.6	37.4 ± 3.9*	18.6 ± 2.5	7.2 ± 2.3*

[a] THP-1wt cells were incubated for 30 min at room temperature with mAbs to TLR2 (clone TL2.1) or TLR4 (clone HTA125) or irrelevant isotype-matched IgG (final concentration, 20 μg/ml). Cells were then assayed for phagocytosis of serum-opsonized GFP-BCG, GFP-*E. coli* and fluorescent latex (F-LTX) beads coated with either LAM (F-LTX-LAM) or LPS (F-LTX-LPS). The proportion of cells ingesting BCG was determined as described in Fig. 2. Values of phagocytosis percentage in cells blocked with control IgG2a were significantly different from those measured in cells blocked with specific Abs (*$p < 0.05$). The data shown are the mean ± SD of three independent experiments.

sense treatment had no effect on LBP-dependent phagocytosis, presumably because in the absence of serum, CR3 was not involved. To verify that the inhibitory effect of antisense was unrelated to any change in the abundance of mycobacterial receptors, surface expression of CD14 and CR3 was measured by flow cytometry. Fluorescence histograms in Fig. 7*C* indicate that antisense treatment resulted in negligible changes (~5% reduction) in surface expression of CD14 and CR3 and these results were insufficient to explain the marked attenuation of CR3-mediated phagocytosis brought about by antisense oligos directed at cytohesin-1.

LAM induced a PI3K-dependent association between cytohesin-1 and CR3

To investigate further the role of cytohesin-1 in CR3-dependent BCG uptake, the effect of LAM on cytohesin localization was examined. Cells were exposed to LAM for 20 min then washed



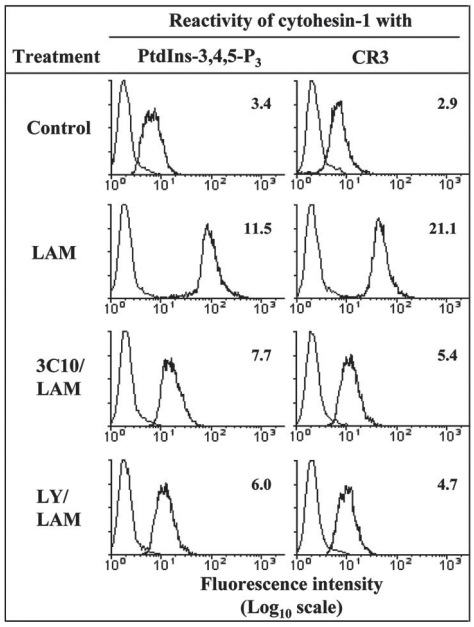


FIGURE 8. Cytohesin-1 colocalization with clustered CR3 in cells stimulated with LAM. *A*, THP-1wt cells were untreated or treated with either LY294002 or anti-CD14 mAb (clone 3C10). LAM (5 μg/ml) was then added in the presence of LBP (200 ng/ml) for 20 min at 37°C. Cells were washed with binding buffer and stained with FITC-conjugated anti-CR3 mAb (clone VIM12) for 20 min at room temperature. After two washes, CR3 surface molecules were cross-linked with unlabeled goat anti-mouse IgG for 20 min at room temperature. Cells were then plated on coverslips, fixed, permeabilized, and stained for endogenous cytohesin-1 with 7H2 rat mAb. Bound Ab was detected with Texas Red-conjugated goat anti-rat IgG. Labeled cells were analyzed with digital confocal microscopy for green and red fluorescence. The images are displayed in red (cytohesin-1) and green (CR3) and yellow signals, the latter indicating colocalization of cytohesin and CR3. Values (*right column*) are mean ± SD of negative/minimal (*a*, *c*, and *d*) or strong positive (*b*) colocalization of 45 cells from three individual experiments. Bar represents 10 μm. *B*, Control and cells preincubated with either LY294002 (LY) or anti-CD14 mAb (clone 3C10) were stimulated with LAM (5 μg/ml) in the presence of LBP (200 ng/ml) for 20 min at 37°C. Cells were lysed in cold Brij 58 buffer and soluble fractions were incubated with 4-μm latex beads coated with anti-cytohesin-1 IgG. Beads were then washed and stained with anti-CR3 or anti-PtdIns-3,4,5-P_3 or irrelevant mAb for 30 min at 4°C. Latex beads were then stained with FITC-labeled secondary Ab and analyzed by flow cytometry. MFI corresponds to the ratio MFI of cells incubated with specific Ab (right histogram) to MFI of cells stained with irrelevant isotype-matched Ab (left histogram). Data shown represent one of three independent experiments with similar results.

and stained with FITC-conjugated anti-CR3 mAb followed by cross-linking with goat anti-mouse Ab. Cells were then plated on coverslips, fixed/permeabilized and incubated with anti-cytohesin-1 mAb followed by Texas Red-conjugated secondary Ab. As shown in Fig. 8*A*, extensive colocalization of cytohesin-1 with clustered CR3 was observed in LAM-treated cells. In contrast, the distribution of cytohesin-1 in control cells was homogeneous and distinct from aggregated CR3. However, cell pretreatment with LY920002 before LAM significantly reduced CR3 association with cytohesin-1, the distribution of which appeared similar to that observed in control cells. Similarly, cells preincubated with CD14 blocking mAb 3C10 inhibited LAM-induced mobilization of cytohesin-1 to clustered CR3 domains.

To demonstrate direct association between cytohesin-1 and CR3 we performed flow cytometry based "pull down" experiments. Latex beads were conjugated to anti-cytohesin-1 Ab and used to pull down cytohesin-1 and attached components to it from lysates of activated cells. Beads were then stained with specific Ab to PtdIns-3,4,5-P_3 or CR3 and processed by flow cytometry to detect their binding to cytohesin-1. The MFI deducted from fluorescence histograms (Fig. 8*B*) showed that, compared with control cells, LAM stimulation increased the level of PtdIns-3,4,5-P_3 attached to cytohesin-1 (3.5-fold increase) concomitant to increased recruitment of CR3 (6.5-fold increase). Pretreatment of cells with PI3K inhibitor or CD14 blocking mAb before LAM stimulation attenuated the recruitment of both PtdIns-3,4,5-P_3 and CR3 to cytohesin-1. Taken together, the results presented in Figs. 7 and 8 suggest a direct role for a CD14/PI3K/cytohesin-1 signaling pathway resulting in enhanced CR3-dependent phagocytosis of BCG.

Discussion

The present study investigated the hypothesis that binding of mycobacteria to macrophages modulates the activity of cell surface receptors so as to enhance bacterial internalization. Using transfected THP-1 and CHO cells we provided clear evidence that CD14 plays a major role in phagocytosis of *M. bovis* BCG and this requires serum factors including LBP. These findings are consistent with a previous report showing that CD14 mediates uptake of mycobacteria by monocyte-derived microglial cells (37). Although there is one report suggesting that CD14 may not be involved in mycobacterial ingestion (38), this study was done in serum-free conditions (no LBP available) and thus such a finding was not unexpected.

Mycobacterial uptake can occur through CD14 alone, but optimal uptake requires the presence of both CD14 and CR3. The dual participation of both CD14 and CR3 in BCG uptake was shown to be not limited to passive and additive roles for each of these receptors. CR3 by itself functioned poorly as a phagocytic receptor for BCG. Indeed, THP-1rsv ($CR3^+/CD14^-$) ingested minimal

amounts of BCG even in the presence of fresh serum. In contrast, addition of fresh serum to THP-1wt cells ($CR3^+/CD14^+$) led to a doubling of BCG uptake when compared with THP-1wt cells cultured without serum, but with LBP. Experiments using blocking mAbs suggested that the provision of complement alone is not sufficient for CR3-dependent phagocytosis; CD14 and a source of LBP must be present as well.

An important question raised by this study was the mechanism of communication between CD14 and CR3. PI3K has been demonstrated to have a direct role in a regulating a range of leukocyte functions dependent upon rearrangement of the cytoskeleton such as adhesion (39), phagocytosis (29, 40), and phagosome biogenesis (26, 41, 42). These properties, together with the knowledge that the activities of β_2 integrin receptors can be modulated through PI3K (15, 16) identified this lipid kinase as an attractive candidate for regulating cross-talk between CD14 and CR3 in the context of the binding and uptake of mycobacteria. Indeed, at least three lines of evidence indicate that this prediction was correct: 1) incubation of cells with either BCG or LAM induced a rapid increase in PI3K activity, 2) the structurally unrelated PI3K inhibitors wortmannin and LY294002 both attenuated phagocytosis of BCG, and most importantly 3) both LAM and mycobacterial cell wall fractions induced a PI3K-dependent expression of an activation epitope on CR3 with increased avidity for the mycobacterial surface.

Extensive investigations aimed to explain how CD14 mediate cell responses revealed TLR2 and TLR4 as important cell surface signaling coreceptors for CD14. Indeed, CD14 is anchored in the cell membrane through a glycosylphosphatidyl tail (43), and it is unable by itself to initiate cell signaling leading to changes in phagocytic or other cell functions. TLR4 has been shown to be essential for recognition of Gram-negative bacteria (44), whereas TLR2 plays a key role in cell responsiveness to components of Gram-positive bacteria and mycobacteria including peptidoglycans, lipoteichoic acid, lipoproteins, and LAM (45–50). These considerations prompted us to examine the hypothesis that CD14/CR3 and PI3K-dependent uptake of BCG might involve TLR2-dependent signaling. In fact, direct evidence for a requirement for TLR2 in phagocytosis of mycobacteria was demonstrated using transfected CHO cells. Such transfectants are considered valid and highly useful models to study macrophage functions including phagocytosis (19, 51) and wild type CHO cells express a nonfunctional TLR2 (52). Coexpression of both CD14 and TLR2 (CHO/CD14/TLR2) markedly enhanced uptake of BCG consistent with the finding that blocking anti-TLR2 but not anti-TLR4 mAbs significantly inhibited phagocytosis of BCG by THP-1wt. These findings added to the observation that PtdIns-3,4,5-P_3 accumulated in the phagocytic cup surrounding BCG in CHO/CD14/TLR2 strongly support the hypothesis of requirement of CD14/TLR2-dependent PI3K activation to initiate the uptake of mycobacteria. They are also consistent with enhanced NF-κB activation in CHO/CD14/TLR2 (but not CHO/CD14 or CHO/TLR2) in response to zymosan (19) and the observation that exposure of $CD14^+$ THP-1 to *Staphylococcus aureus* resulted in tyrosine phosphorylation of TLR2 and its physical association with PI3K (53). In the latter study, it was also shown that stimulation of TLR2 led to activation of the Rho family GTPases Rac1 and Cdc42, which are key regulators of cytoskeletal reorganization (54, 55). Therefore, CD14, although devoid of an intracytoplasmic signaling domain, would be able to trigger the necessary cytoskeletal force to internalize mycobacteria by activating a TLR2/PI3K-dependent signaling pathway.

A key question raised by these findings was the molecular basis by which activation of CD14/TLR2 and PI3K-dependent signaling led to modulation of the properties of CR3. We and others have shown that cytohesin-1 is a key regulator of leukocyte adhesion with which its interaction with the cytoplasmic tail of CD18 brings about increased avidity of LFA-1 (CD18/CD11a) for ICAM-1 (12, 16, 56). Cytohesin-1 binds to and regulates β_2 integrins and this process is positively regulated by the binding of the PI3K metabolite, PtdIns-3,4,5-P_3, to the cytohesin-1 pleckstrin homology domain (13, 36). We found several lines of evidence to support a role for cytohesin-1 in CR3-dependent phagocytosis of BCG: 1) the uptake of serum- but not LBP-opsonized BCG was significantly attenuated in THP-1wt cells with knockdown cytohesin-1, 2) intracellular staining showed a CD14- and PI3K-dependent colocalization of cytohesin-1 with CR3 in LAM-stimulated cells, and 3) pull-down experiments detected the physical association between cytohesin-1 and either PtdIns-3,4,5-P_3 and CR3 in LAM-stimulated cells. This role for cytohesin-1 is also consistent with the observation that activated cytohesin-1 is able to tightly associate with the actin cytoskeleton (14).

The PI3K/cytohesin-1-mediated activation of CR3 we describe represents a novel and additional mechanism of CR3 activation to previously described functional associations between CR3 and several GPI-linked proteins, including, FcγRIIIB (57), CD87 (58), and CD14 by itself (59). Such receptor clustering appears to occur in a dynamic microenvironment provided at the cell surface by lipid raft microdomains, which facilitate ligand-specific cellular responses initiated by GPI-linked molecules (60).

The conclusion that BCG enhances its internalization by co-opting host surface receptors is reminiscent of what has been described for phagocytosis of *Bordetella pertussis*, the causative agent of whooping cough. The uptake of *B. pertussis* by monocytes has been shown to be regulated by an Arg-Gly-Asp site in the bacterial surface hemagglutinin, which enhances *B. pertussis* binding to CR3. This enhancement is regulated by PI3K and requires a signal transduction complex, composed of a leukocyte β_3 integrin ($\alpha_v\beta_3$) and integrin-associated protein CD47 (61, 62). Furthermore, a recent report (63) suggested that FcγR stimulation promotes a PI3K-dependent CR3 clustering into high avidity receptor in the phagocytic cup of latex beads. Taken together, these findings suggest that modulation of integrin function by PI3K may be involved in regulating the uptake of diverse microbes.

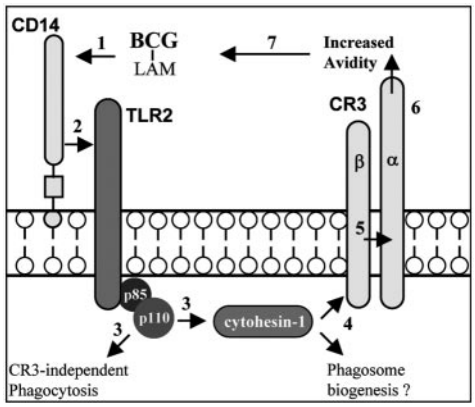


FIGURE 9. Cooperativity between CD14 and CR3 mediates optimal ingestion of BCG. Signaling through membrane-bound CD14 initiated by BCG or LAM leads to TLR2-mediated activation of PI3K. The latter is responsible for triggering two pathways for bacterial internalization: a unique CD14 pathway that can function in the absence of CR3 and a cytohesin-1-regulated CR3-dependent pathway that together with CD14 results in optimal phagocytosis of BCG.

It is of interest to view the results of the present study in the context of those of Tailleux et al. (64), who reported that *M. tuberculosis* enters human dendritic cells after binding to the recently identified lectin dendritic cell-specific ICAM-3 grabbing nonintegrin. This study found that CR3 was not involved in mycobacterial binding to dendritic cells. Given that dendritic cells gradually lose their surface CD14 during the maturation process (65) it is reasonable to consider that CR3 remains inactive in these cells in the absence of a CD14-dependent regulatory mechanism, in contrast to our findings with $CR3^+/CD14^+$ THP-1 cells.

In summary, the results presented in this study suggest a dynamic model that regulates the uptake of mycobacteria by macrophages (Fig. 9). In this model, binding of BCG or LAM to CD14 triggers signaling through TLR-2 leading to activation of PI3K. This creates a point of bifurcation where in one direction based upon the effects of PI3K on the cytoskeleton alone, suboptimal levels of CD14-dependent bacterial ingestion can proceed independently of CR3. In the other direction, through the action of the PI3K product PtdIns-3,4,5-P_3, the molecular adaptor cytohesin-1 is recruited to the membrane where it binds to the β-chain of CR3 and converts it into an active receptor for BCG. Thus, CD14-dependent activation of PI3K through TLR2 leads to recruitment of a second receptor that acts cooperatively to bring about optimal bacterial internalization.

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Disclosures

The authors have no financial conflict of interest.

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