Depletion of cellular cholesterol enhances macrophage MAPK activation by chitin microparticles but not by heat-killed *Mycobacterium bovis* BCG

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MBCD-treated RAW 264.7 MØ. We found that the initial recognition and internalization of chitin particles were not significantly different between untreated and MBCD-treated MØ. However, in MBCD-treated MØ compared with untreated MØ, the initial activation of all MAPK family members was significantly accelerated and enhanced by chitin microparticles. The initial phase of MAPK phosphorylation in MBCD-treated MØ, the initial activation of all MAPK family members was observed. However, in MBCD-treated MØ compared with untreated MØ, recognition and internalization of chitin particles were not significantly different between untreated and MBCD-treated MØ. We found that the initial activation of all MAPK family members was observed. However, in MBCD-treated MØ compared with untreated MØ, recognition and internalization of chitin particles were not significantly different between untreated and MBCD-treated MØ.

**Materials and Methods**

Reagents and antibodies. Chitin powder was purchased from Sigma (St. Louis, MO), and 1- to 10-μm and >50-μm chitin particles and 1- to 10-μm chitosan (de-acetylated chitin) particles were prepared as described previously (18, 30). Soluble chitin oligosaccharide was provided by Stylochon Technologies (Chiba, Japan). Latex beads (1.1 μm, polystyrene), MBCD, and arachidonic acid (AA) were purchased from Sigma. CpG-ODN (5'-TCC ATG ATC TTC CTC AGC TT-3'; unmethylated) with a phosphorothioate backbone was purchased from TriLink (Sorrento Mesa, CA). The cultured bacteria of *M. bovis* BCG Tokyo 172 strain were washed, autoclaved, and lyophilized (29). All stimulating reagents were suspended in endotoxin-free saline at 10 mg/ml stock solutions, and aliquots were stored at −80°C. MBCD was a 0.5 M stock solution in endotoxin-free saline, and AA, as a 100 mg/ml stock solution in 100% ethanol were stored at −80°C until use. Rabbit polyclonal antibodies (Abs) against MAPK (anti-p38, anti-ERK1/2, and anti-JNK) and dual-phosphorylated MAPK (anti-p-p38, anti-p-ERK1/2, and anti-p-JNK) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-COX-2 Ab was purchased from Cayman Chemicals (Ann Arbor, MI). Rat monoclonal Abs against F4/80, Mac-1, Fcγ receptor II/III (FcγR), scavenger receptor A (SR-A; 2F8), and Toll-like receptor 4 (TLR4) were purchased from BD Biosciences (San Diego, CA). Rabbit polyclonal anti-mannose receptor (MR) Ab was a gift from Dr. Philip Stahl, Washington University (St. Louis, MO).

Cholesterol depletion with MBCD. Murine MØ-like RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were grown and maintained in RPMI 1640 containing 5% heat-inactivated fetal bovine serum (FBS) as described previously (18). For all experiments testing MAPK activation, MØ were incubated in serum-free RPMI 1640 at 37°C for 2 h to achieve serum starvation before MBCD treatment. To deplete cholestérol, MØ were incubated with 0 (saline), 1, or 5 mM MBCD at 37°C for 1 h, before chitin particle stimulation. Cell viability was determined by trypan blue exclusion and lactate dehydrogenase (LDH) release according to the manufacturer’s instructions. (Cytotoxicity Colorimetric Assay Kit, Oxford Biomedical Research, Oxford, MI).

Cytometric detection of phagocytosed chitin particles and MØ surface antigens. For chitin binding and phagocytosis assays (33), 1- to 10-μm chitin particles were labeled with fluorescein isothiocyanate (FITC). Particles (10 μg/ml) and FITC (0.1 mg/ml) were mixed and incubated in 0.1 M NaHCO₃ at 22°C for 2 h. Glycine (final 1 M) was added to bind free FITC, after which labeled particles were washed and suspended in saline at 10 mg/ml. To assess cell-surface binding of chitin, MØ were incubated with 100 μg/ml FITC-chitin particles on ice for 30 min. Free particles were removed by being washed three times, and cellular fluorescence was measured cytomorphologically (BD FACSCalibur system with CELL Quest acquisition plus analysis program; Becton-Dickinson Immunocytometry Systems, San Jose, CA). To confirm that MØ binding to chitin particles was not altered by FITC, an excess of unlabeled particles (1,000 μg/ml) was used to compete with FITC-chitin.

For evaluation of phagocytosis, MØ were incubated with 100 μg/ml FITC-chitin particles at 37°C for 20 or 40 min. Fluorescence of unphagocytosed FITC-chitin was quenched with 50 mM acetate-buffered saline (pH 4.5) containing 2 mg/ml trypan blue, and the fluorescence intensity of MØ with intracellular FITC-chitin particles was measured cytomorphologically. The presence of intracellular FITC-chitin particles was further confirmed by fluorescence microscopy (Provis AX70 Microscope with MagnaFire, Olympus, Center Valley, PA).

Expression of F4/80, Mac-1, FcγR, SR-A, TLR4, or MR on MØ was determined cytometrically as indicated previously (28).

**MAPK activation.** Western blot analyses of MAPK phosphorylation were performed as described previously (18). Briefly, MØ (10⁶/ml) were stimulated with each agonist or saline at 37°C for 0, 10, 20, 30, or 40 min. After cell lysis, equal amounts of cellular protein were separated by SDS-PAGE using SDS-11% polyacrylamide gel and then electroblotted onto polyvinylfluoride membranes. After the membrane was blocked with nonfat dry milk, proteins were stained with primary Ab (anti-p38, anti-ERK1/2, anti-JNK, anti-p-p38, anti-p-ERK1/2, or anti-p-JNK) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Stained bands were detected by chemiluminescence (ECL Western Blotting Detection Reagents, Amershams Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. Intensity of specific bands was quantified digitally using graphic imaging software (NIH Image 1.5).

Isolation of particle-associated cellular proteins. MBCD- or saline-treated MØ (2 × 10⁶/ml) were stimulated with 100 μg/ml 1- to 10-μm chitin particles at 37°C for 10, 20, or 40 min, washed with saline, suspended in homogenization buffer (50 mM Tris·HCl, pH 7.5, 0.32 M sucrose, 10 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA, 1:500 protease inhibitor cocktail; Sigma), and homogenized by sonication (20 s). Particles were isolated from lysates by centrifugation (400 g, 4°C, 10 min) and washed five times with saline. Proteins associated with the particles were extracted with SLS-lysis buffer by heating at 95°C for 5 min. Total and phosphorylated p38 and ERK1/2 were quantified by Western blot analysis with specific antibodies as described above. Typically, −1 μg chitin-associated protein was isolated at 20 min from 10⁷ saline-treated MØ. The recovery rates in this study were comparable for samples with or without MBCD treatment.

Cytokine production. MØ (5 × 10⁶/ml) were stimulated with agonist or saline at 37°C for 3 h (for TNF-α) and 24 h (for IL-10). TNF-α and IL-10 levels in culture supernatants were measured by specific two-site ELISA (BD Biosciences). Experiments were performed in triplicate with triplicate assays for each experiment. To permit comparison of all experimental data for each experiment, results were normalized to the mean response for the highest agonist concentration in the absence of MBCD.

IL-10 mRNA expression. MØ (5 × 10⁶/ml) were stimulated with agonist or saline at 37°C for 6 and 24 h. Total RNA was extracted from the cells with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. IL-10 mRNA expression was examined by RT-PCR. Reverse transcription of mRNA was achieved by SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo-(dT) primer according to the manufacturer’s instructions. PCR primers used were IL-10 (forward: 5'-GGT TGC CAA GCC TTA TCG GA-3', reverse: 5'-ACC TGG TTC ACT GTC TGG CT-3') and GAPDH (forward: 5'-TTC ACC ACC ATG GAG AAG GC-3', reverse: 5'-GGC ATG GAC TGT CAT CA-3'). PCR products (15 μl) were electrophoresed on 2% agarose gel. After ethidium bromide staining, PCR products were visualized by ultraviolet illumination.

COX-2 production and PGE₂ release. MØ (5 × 10⁶/ml) were stimulated with each agonist or saline at 37°C for 2 h. COX-2 in cell lysates was analyzed by Western blot analysis using anti-COX-2, as described previously (18).
Results

Cholesterol depletion of MØ does not significantly alter initial phagocytosis of chitin particles. To investigate the effects of cholesterol depletion on MØ viability, RAW 264.7 cells were treated with 1, 5, 7.5, 10, 15, or 20 mM MBCD. Treatment of MØ with 5 mM MBCD, which has been used to inhibit phagocytosis of intracellular bacteria (17, 39), in the presence or absence of 5% heat-inactivated FBS reduced cellular cholesterol levels to 40–45% of that present before treatment (Fig. 1A). At this concentration or less, MBCD did not reduce cell viability at 24 h (Fig. 1B).

We determined the effect of cholesterol depletion on expression of the selected MØ surface antigens F4/80, Mac-1, FcγR, SR-A, TLR4, and MR. As shown in Table 1, these antigens were constitutively expressed, respectively, by 61, 85, 71, 85, 80, and 86% of RAW264.7 cells. The expression of F4/80 was slightly increased (Table 1; 69% of MØ), whereas Mac-1, FcγR, and SR-A were slightly reduced (81, 65, and 81% of MØ, respectively) by treatment with 5 mM MBCD. The expression of TLR4 and MR were not altered by MBCD. The values for mean fluorescence intensity were consistent with the effects of MBCD on the expression of MØ antigens (Table 1). Therefore, MBCD at 5 mM or less was used for further experiments. All studies of chitin stimulation in the presence of MBCD were terminated within 6 h.

We also determined whether cholesterol depletion modifies MØ binding and internalization of FITC-chitin microparticles. As shown in Fig. 2A, 80% of saline-treated MØ bound to FITC-chitin particles, which was not significantly altered by MBCD treatment. This binding was completely inhibited in the presence of nonlabeled chitin particles but not latex beads (data not shown). Internalization of FITC-chitin particles at 37°C was analyzed following quenching of unphagocytosed FITC-chitin. As shown in Fig. 2B, the magnitude of phagocytosis is similar for MBCD- and saline-treated MØ at 20 min (12% and 12%, respectively).Saline-treated MØ may have internalized more particles than MBCD-treated MØ at 20 min, since the peak fluorescence intensity was slightly reduced by MBCD treatment (Fig. 2B). However, phagocytosis at 40 min was significantly reduced in MBCD-treated MØ [33 ± 3 and 21 ±

Table 1. Effects of MBCD on surface expression of MØ antigens

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Macrophages (MØ) were treated with saline or 5 mM methyl-β-cytodextrin (MBCD) for 1 h at 37°C. *Data shown are percentages of positive cells reacting to primary antibodies listed, from which percentage of cells stained with secondary antibody alone has been subtracted (see Fig. 2). MFI, mean fluorescence intensities. The second antibody controls of 0 and 5 mM MBCD treatments were both 7.0. The data shown are representative of three independent experiments. FcγR, Fcγ receptor II/III; SR-A, scavenger receptor A; TLR, Toll-like receptors; MR, mannose receptor.

For PGE2 release, cells treated with chitin were further incubated in serum-free RPMI 1640 with 1 μg/ml AA or saline at 37°C for an additional 2 h. Culture supernatants were harvested and stored at −80°C. PGE2 levels were assayed by ELISA (Cayman Chemicals) (18). Experiments were performed in triplicate with triplicate assays for each experiment. The results were analyzed as described above for cytokines.

Cellular cholesterol level. Cholesterol was extracted from cell pellets (10⁶ cells) with methanol-chloroform (2:1), followed by addition of an equal volume of chloroform-water (1:1). Cholesterol was recovered from the chloroform layer by lyophilization. Extracted lipids were dissolved in the buffer for Cholesterol E test (Wako Bioproducts, Richmond, VA), and cholesterol in the extract was determined by enzymatic colorimetric assay, according to the manufacturer’s instructions. Cellular protein was measured, as described previously (18), and cellular cholesterol levels were normalized to the protein levels. Normalized cholesterol levels of saline-treated MØ were considered as 100%.

Endotoxin removal. Endotoxin was removed from soluble materials for culture by filtration and sterilization through a 0.22-μm Zetapore membrane (AMF-Cuno; Cuno, Meriden, CT) (22). Chitin particles and HK bacteria were suspended in and washed with endotoxin-free saline. The final preparations were monitored for endotoxin by the amebocyte assay (Sigma) (27). No endotoxin was detected in suspensions of chitin particles or HK-BCG.

Statistics. Differences between mean values were analyzed by Student’s t-test. P < 0.05 was considered statistically significant.

Fig. 1. Effects of methyl-β-cytodextrin (MBCD) treatment of RAW 264.7 cells on cellular cholesterol level and cell viability. RAW264.7 cells (10⁶ cells/ml) were suspended in serum-free RPMI 1640 or RPMI 1640 containing 5% fetal bovine serum and treated with MBCD at the indicated concentrations or saline at 37°C. A: after 1-h incubation with 5 mM MBCD, cellular cholesterol and protein were extracted. Cellular cholesterol and protein levels were measured by enzymatic colorimetric assay and BCA reagent, respectively. Cellular cholesterol level was normalized to the cellular protein level. Normalized cholesterol levels of saline-treated MØ were considered as 100%. Values are means ± SE; n = 3; *P < 0.0001 when compared with saline-treated MØ. B: after 24-h incubation, viability of MØ was determined by LDH release into culture supernatants. Viability of saline-treated MØ was considered as 100%. The data shown are representative of three independent experiments. Values are means ± SE; n = 3.

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1% (means ± SE, n = 3, P < 0.01) for saline- and MBCD-treated MØ, respectively.

Other particles including HK-BCG, 1- to 10-μm chitosan, and 1.1-μm latex beads were phagocytosed by MBCD-treated or untreated MØ, and phagocytic capacities were not changed 40 min after particle exposure (data not shown).

Chitin-induced MAPK phosphorylation is accelerated and enhanced by cholesterol depletion. We demonstrated that when MØ phagocytosed chitin particles, MØ MAPK families including p38, ERK1/2, and JNK were phosphorylated (18). Treatment of MØ with MBCD alone at 1 or 5 mM resulted in no significant phosphorylation of p38, ERK1/2, or JNK during the experimental period (Fig. 3). As shown in Fig. 3, at 20 min after chitin particle stimulation, the levels of phosphorylated p38 and ERK1/2 in MBCD-treated cells were markedly increased compared with those in saline-treated MØ. At 40 min, the magnitudes of ERK1/2 activation in MBCD-treated MØ decreased but for p-p38 were still higher than for saline-treated MØ phagocytosing chitin particles (Fig. 3). The enhanced phosphorylation of each MAPK was greater at 5 mM than at 1 mM MBCD (Fig. 3). We also found accelerated and enhanced phosphorylation of JNK with kinetics similar to those of p-p38 in MBCD-treated MØ (data not shown).

The effects of MBCD were relatively selective for chitin microparticles, since MAPK activation by other agonists, CpG-
ODN, and HK-BCG was relatively insensitive to the treatment with MBCD (Fig. 4).

We have previously found that cellular activation in response to chitin occurs specifically with phagocytosable particles but not soluble chitin oligosaccharide, nonphagocytosable chitin particles (>50 μm), 1- to 10-μm chitosan particles, or 1.1-μm latex beads (18). We observed that for other than phagocytosable chitin microparticles, there was no effect of MBCD treatment on MØ activation (Fig. 5).

Phosphorylation of p38 and ERK1/2 associated with intracellular chitin particle is accelerated and enhanced by cholesterol depletion. We next determined the extent of phosphorylation of MAPK associated with intracellular chitin particles in MBCD-treated and saline-treated MØ. Cellular proteins associated with chitin particles were isolated from MBCD- and saline-treated cells 10, 20, and 40 min after phagocytosis of chitin particles. After fusion with late endosomes/lysosomes, mature phagosomes express LAMP-1 (25). The levels of LAMP-1 in whole cell lysates were comparable for saline- and MBCD-treated cells (Fig. 6). MAPKs p38 and ERK1/2 and the respective phosphorylated forms were detected in the particle-associated fractions at 10, 20, and 40 min (Fig. 6). The relative amounts of chitin-associated phosphorylated MAPK were greater in the MBCD- than those in saline-treated cells. Phosphorylated JNK was not detected in the particle-associated fraction (data not shown).

Cholesterol depletion enhances chitin-induced TNF-α production and COX-2-mediated PGE2 synthesis. Although treatment with MBCD alone did not result in observable MAPK activation, 5 mM MBCD enhanced slightly but significantly TNF-α production. Treatment with 1 mM MBCD resulted in 3- and 1.8-fold increases in TNF-α production in response to 20 and 100 μg/ml chitin particles, respectively. There was similar enhancement for MØ treated with 5 mM MBCD (Fig. 7A). In response to CpG-ODN, or HK-BCG, TNF-α production tended to increase following cholesterol depletion, but the changes were not significant (Fig. 7A).

COX-2 expression was induced by treatment with 5 mM MBCD alone and was further enhanced by 100 μg/ml chitin particles (Fig. 7B). After treatment with 1 mM MBCD, increased chitin particle-induced COX-2 expression is associated with increased PGE2 release. As shown in Fig. 7C, chitin particle-activated MØ released 233 ± 11 and 506 ± 52 pg/ml PGE2 in the absence and presence of 1 mM MBCD, respectively. However, for MØ treated with 5 mM MBCD, the release of PGE2 was not different from that of MØ treated with saline despite the increased COX-2 expression (Fig. 7, B and C). After treatment with 5 mM MBCD, nuclear envelope localization and COX-2 enzyme activity were intact (data not shown). Therefore, additional factors that may include phospholipase A2, PGE synthases, and/or the assembly of these enzymes at membrane sites may be impaired by cholesterol depletion with 5 mM MBCD. CpG-ODN also induces COX-2-mediated PGE2 biosynthesis (18). The treatment with 1 mM MBCD did not enhance CpG-ODN-induced COX-2 expression (Fig. 7B) or PGE2 release (data not shown).

Effects of cholesterol depletion on chitin-induced IL-10 production. Despite the release of TNF-α and PGE2, which are both endogenous inducers of IL-10 (7, 31, 34), we found previously that chitin particles do not induce IL-10 expression by MØ at 24 h (18) nor was IL-10 mRNA detected (Fig. 8). In contrast, CpG-ODN-induced expression of IL-10 was significantly increased at 24 h, as described previously (18) with mRNA synthesis observed (Fig. 8). We further determined the effect of cellular cholesterol depletion on chitin particle-induced IL-10 mRNA expression. As shown in Fig. 8, MBCD treatment before exposure to chitin particles did not result in IL-10 mRNA expression at 6 h. CpG-ODN-induced IL-10 mRNA was not increased by MBCD treatment (Fig. 8). Taken together, our results indicate that following cholesterol depletion, the Th1 adjuvant chitin does not induce IL-10 mRNA expression, despite effects on MAPK phosphorylation, TNF-α production, and PGE2 biosynthesis.

DISCUSSION

Administration of chitin microparticles in mouse models of asthma and mycobacterial infections promotes MØ activation leading to the downregulation of allergic responses and the enhancement of host immunity against mycobacteria, respectively (26, 29), suggesting that these particles may be a unique and clinically useful Th1 adjuvant. Our previous (18, 30) and present studies clearly indicate that both recognition and phagocytic entry of chitin microparticles are required for MØ activation characterized by MAPK phosphorylation, Th1 cyto-
kine production, and COX-2-mediated PGE2 biosynthesis. The depletion of cholesterol from the plasma membrane results in only minimal alteration in the recognition and internalization of chitin particles or HK-BCG within 20 min after particle stimulation (Fig. 2). However, the rate and extent of chitin particle-induced MAPK phosphorylation are accelerated and enhanced, respectively, for MBCD-treated MØ compared with saline-treated MØ (Fig. 3). This is also detected in phagosomes containing chitin microparticles (Fig. 6). In contrast, only slight upmodulation of MAPK phosphorylation following depletion of cholesterol occurred following stimulation with HK-BCG or CpG-ODN (Fig. 4). These results indicate that phosphorylation of MAPKs in the MØ response to chitin microparticles, but not to HK-BCG or CpG-ODN, is enhanced by depletion of membrane cholesterol.

Our previous studies with MØ (18, 27, 30) clearly indicate that phagocytosis of chitin microparticles (1–10 μm), but not a soluble form of chitin or >50 μm chitin particles, promotes MAPK activation and Th1 responses. The present study further indicates that, in contrast to results for phagocytosable chitin microparticles (1–10 μm), MAPK activation in response to >50-μm chitin particles, soluble chitin, 1- to 10-μm chitosan particles, or 1.1-μm latex beads is not altered by MBCD treatment of MØ. Thus requirements for recognition and response to chitin preparations and latex beads are not qualitatively altered by MBCD treatment. GlcNAc residues are recognized by mannose-type C-type lectin-like receptors (CLR) including MR, endo-180, and dendritic cell-specific ICAM-grabbing nonintegrin (DC-SIGN), which is located in the DRM of dendritic cells (16, 24). Although the receptor(s), other than MR, that contribute to phagocytosis of chitin particles, has not been completely identified, MBCD treatment does not change the capacity of RAW264.7 cells for chitin binding or phagocytosis at 20 min (Fig. 2).

It is likely that cellular signals for both MAPK phosphatase activation and attenuation are also induced by chitin. Our results are consistent with either enhanced activity of MAPK kinases (MAPKKs) or the inhibition of MAPK phosphatase (MKP) activity following disruption of the integrity of DRM by MBCD. Previous studies suggest that DRM-associated regulation of cellular signaling may depend on caveolin-, oxysterol-binding protein-, and phosphatidylinositol-3 kinase-mediated mechanisms (3, 8, 9, 12, 21, 37, 38). Further studies to determine whether these molecules are involved in chitin-induced MØ activation are underway.
Another finding is that depletion of MØ cholesterol results in significant enhancement of chitin microparticle-induced MØ production of TNF-α, a Th1 cytokine (Fig. 7). In MØ treated with 1 mM MBCD, TNF-α production and COX-2-mediated PGE₂ biosynthesis are significantly increased. In response to chitin particles, the production of PGE₂ has a biphasic dependence on cellular cholesterol levels. After treatment with 1 mM MBCD and chitin particle stimulation, the increase in PGE₂ correlates with the increase in COX-2 expression. However, chitin particles do not induce IL-10 mRNA (Fig. 8) or IL-10 (data not shown), in agreement with our previous report (18), despite the fact that chitin particles induce PGE₂ and TNF-α that potentially promote IL-10 production (7, 31, 34). This study provides further support for a MAPK-independent mechanism for stimulation of IL-10 production.

In addition to TNFα and IL-10, RAW264.7 cells produced detectable levels of IL-1β, IL-6, and GM-CSF in response to chitin microparticles (data not shown). For RAW264.7 cells stimulated with LPS, p38 and ERK1/2 are phosphorylated followed by production of these cytokines (15, 35). Our preliminary studies indicated that chitin particle-induced production of these cytokines is enhanced in MBCD-treated cells (data not shown). Further studies will be required to determine whether production of other cytokines following membrane cholesterol depletion is correlated with their dependence on MAPK phosphorylation.

In contrast to stimulation by chitin microparticles, MAPK activation in response to HK-BCG is relatively insensitive to cholesterol depletion. These microbes are recognized by multiple receptors including TLRs and scavenger receptors (1, 16). We have also observed that phagocytosis of these microbes by cytochalasin D does not prevent MAPK activation and Th1 cytokine production (data not shown). In preliminary experiments, we have found that solubilized components of HK-BCG, prepared by filtration of a sonicated HK-BCG prep-

![Fig. 7. Effect of MBCD on chitin particle-induced tumor necrosis factor (TNF)-α production, cyclooxygenase-2 (COX-2) expression, and PGE₂ release. MBCD- and saline-treated MØ were stimulated with indicated doses of agonists. A: levels of TNF-α in culture supernatants 3 h after agonist stimulation were measured by ELISA. Results for each experiment were normalized to the response to the highest agonist concentration for saline-treated cells. Values are means ± SE, n = 3; *P < 0.05; #P < 0.001 when compared with TNF-α induced in saline-treated cells stimulated with same dose of agonist. B: COX-2 levels in the cell lysate 2 h after agonist stimulation were detected by Western blot analysis. C: MØ stimulated with chitin particles or saline for 2 h were further elicited with 1 µg/ml arachidonic acid (AA) for additional 2 h. The levels of PGE₂ in culture supernatants were measured by ELISA and normalized as for A. Values are means ± SE; n = 3. #P < 0.001 when compared with PGE₂ induced in saline-treated cells stimulated with the same concentration of chitin.]

![Fig. 8. Effect of MBCD on IL-10 mRNA expression by chitin particle-stimulated MØ. MØ treated with MBCD at 0 (saline), 1, and 5 mM were stimulated with chitin particles, CpG-ODN, or saline at 37°C for 6 h. After extraction of total RNA, RT-PCR was performed as described in MATERIALS AND METHODS. Levels of IL-10 and GAPDH mRNA are shown. These data are representative of three independent experiments.]}
Phagocytosis through a 0.22-μm Millipore membrane, induced TNF-α production and COX-2 expression (data not shown). These results suggest that phagocytic entry and phagosome formation are not required for MAPK activation by HK-BCG. Furthermore, MAPK activation and Th1 cytokine production induced by CpG-ODN, which is mediated through TLR9 and does not require actin polymerization (13, 18), is not significantly altered by depletion of membrane cholesterol (Fig. 4). Thus MØ activation signals derived from phagocytic entry and phagosome formation in response to chitin microparticles appear to be more sensitive to membrane cholesterol depletion.

In conclusion, although the early phase of phagocytosis of chitin microparticles is comparable for untreated MØ and MØ depleted of cholesterol by MBCD, MAPK activation and Th1 cytokine production in response to chitin is markedly enhanced in MBCD-treated MØ. Our results suggest differential membrane structural requirements for MAPK activation and cytokine or PGE2 production compared with binding and phagocytosis of chitin particles. IL-10 is not produced in response to chitin, and this is not altered by cholesterol depletion. Furthermore, cholesterol depletion does not significantly alter initial stages of chitin phagocytosis or MAPK activation and Th1 cytokine production by HK-BCG. Thus membrane structures integrated by cholesterol appear to be important for the normal regulation of chitin-induced MØ MAPK activation, probably reflecting the role of phagocytic entry and phagosome formation as well as cellular recognition by different surface receptors.

ACKNOWLEDGMENTS

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