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# Increased Mitogen-Activated Protein Kinase Activity and TNF-$\alpha$ Production Associated with *Mycobacterium smegmatis*- but Not *Mycobacterium avium*-Infected Macrophages Requires Prolonged Stimulation of the Calmodulin/Calmodulin Kinase and Cyclic AMP/Protein Kinase A Pathways[1]

**Mahesh Yadav,[2] Shannon K. Roach,[2] and Jeffrey S. Schorey[3]**

**Previous studies have shown the mitogen-activated protein kinases (MAPKs) to be activated in macrophages upon infection with *Mycobacterium*, and that expression of TNF-$\alpha$ and inducible NO synthase by infected macrophages was dependent on MAPK activation. Additional analysis demonstrated a diminished activation of p38 and extracellular signal-regulated kinase (ERK)1/2 in macrophages infected with pathogenic strains of *Mycobacterium avium* compared with infections with the fast-growing, nonpathogenic *Mycobacterium smegmatis* and *Mycobacterium phlei*. However, the upstream signals required for MAPK activation and the mechanisms behind the differential activation of the MAPKs have not been defined. In this study, using bone marrow-derived macrophages from BALB/c mice, we determined that ERK1/2 activation was dependent on the calcium/calmodulin/calmodulin kinase II pathway in both *M. smegmatis*- and *M. avium*-infected macrophages. However, in macrophages infected with *M. smegmatis* but not *M. avium*, we observed a marked increase in cAMP production that remained elevated for 8 h postinfection. This *M. smegmatis*-induced cAMP production was also dependent on the calmodulin/calmodulin kinase pathway. Furthermore, stimulation of the cAMP/protein kinase A pathway in *M. smegmatis*-infected cells was required for the prolonged ERK1/2 activation and the increased TNF-$\alpha$ production observed in these infected macrophages. Our studies are the first to demonstrate an important role for the calmodulin/calmodulin kinase and cAMP/protein kinase A pathways in macrophage signaling upon mycobacterial infection and to show how cAMP production can facilitate macrophage activation and subsequent cytokine production. *The Journal of Immunology,* 2004, 172: 5588–5597.**

*Mycobacterium avium* is a major opportunistic pathogen of AIDS patients in the United States and is responsible for significant morbidity and mortality in HIV-infected individuals. *M. avium* can be either ingested or inhaled and requires the host macrophage for its survival and replication. Numerous studies have shown that macrophages respond to an *M. avium* infection by producing various effector molecules essential for controlling an infection including the cytokines TNF-$\alpha$ and IL-12, as well as reactive oxygen and nitrogen intermediates (1, 2). It is also known that macrophages infected with pathogenic mycobacteria including *M. avium* show limited production of many of these inflammatory mediators relative to macrophages infected with nonpathogenic mycobacteria (3, 4). The panel of cytokines a macrophage produces is dependent on the mode of its activation. Differences in cytokine production have been shown for macrophages stimulated with LPS, zymosan, and Gram-negative and Gram-positive bacteria, as well as following stimulation with various cell wall components of mycobacteria. Following stimulation, many signal transduction pathways are activated that regulate transcription factors important in cytokine transcription. However, mycobacterial regulation of the signal transduction cascades important in cytokine production is poorly understood.

The mitogen-activated protein kinase (MAPK)[4] cascade is one such signaling system that is activated upon mycobacterial infection and has been implicated in mycobacterial pathogenesis. The MAPKs are a family of protein kinases that are composed of the extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38, and stress-activated protein kinase/c-Jun N-terminal kinase pathways. The MAPKs are highly conserved serine-threonine kinases and are activated by distinct upstream MAPK kinases through dual phosphorylation of their Tyr-XXX-Thr motif (5). Recent studies have shown the MAPK cascades to be differentially activated in macrophages upon infection with pathogenic and nonpathogenic mycobacteria. We have shown that nonpathogenic, fast-growing mycobacteria such as *Mycobacterium smegmatis* induce a more sustained activation of the MAPKs p38 and ERK1/2 in primary murine bone marrow-derived macrophages (BMM$\phi$) when compared with *M. avium* infection. This activation of MAPKs was necessary for the high levels of TNF-$\alpha$ produced during an *M.*

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[4] Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; BMM$\phi$, bone marrow-derived macrophages; CaM, calmodulin; CaMK, $Ca^{2+}$-calmodulin-dependent protein kinase; PKA, protein kinase A; AC, adenylate cyclase; NHS, normal human serum; LAM, lipoarabinomannan; RC, resting cell.

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*smegmatis* infection (6). However, these studies did not address whether mycobacteria differentially regulate the MAPKs directly, or whether there are upstream signaling pathways activated initially upon infection that are responsible for differences in MAPK activation.

Two pathways upstream of the MAPKs that have been implicated in the regulation of a macrophage immune response are the cAMP/protein kinase A (cAMP/PKA) and $Ca^{2+}$/calmodulin/$Ca^{2+}$-calmodulin-dependent protein kinase ($Ca^{2+}$/CaM/CaMK) pathways (7–10). The cAMP/PKA pathway is activated following the synthesis of cAMP by adenylate cyclase (AC). cAMP binds to PKA, activating the kinase's catalytic subunits, which can then phosphorylate a wide variety of proteins including other kinases, transcription factors, and phosphatases (11). The cAMP/PKA pathway has been shown to both activate and suppress many inflammatory cytokines (12, 13). Similarly, the CaM/CaMK pathway is important in the activation of numerous transcription factors implicated in immune regulation including ELK-1, c-JUN, and ATF-2 (14). The CaM/CaMK pathway is activated following an increase in intracellular $Ca^{2+}$ levels. Cytosolic $Ca^{2+}$ binds to CaM, which in turn activates downstream kinases such as CaMKII. Both the cAMP/PKA and CaM/CaMK pathways have been shown to be upstream of MAPK activation and important in an inflammatory response and therefore were good candidates for regulating macrophage MAPK activity following the mycobacterial infections.

In the present study, we found that macrophages, infected with either *M. smegmatis* or *M. avium* 724, have activated CaM/CaMK and cAMP/PKA pathways, and that these signaling molecules were upstream of ERK1/2 activation. However, cAMP production was maintained at elevated levels in *M. smegmatis*- compared with *M. avium*-infected macrophages, and the sustained ERK1/2 activation and increased TNF-$\alpha$ production observed in *M. smegmatis*-infected cells was dependent on CaM/CaMK and cAMP/PKA activation. These studies highlight the importance of CaMK and cAMP in the macrophage signaling response to a mycobacterial infection and demonstrate a novel role for cAMP and PKA in maintaining macrophage activation following an infection with nonpathogenic *M. smegmatis*.

## Materials and Methods

### *BMM$\phi$ isolation and culture*

BMM$\phi$, used in all experiments, were isolated from 6- to 8-wk-old BALB/c mice as previously described (6). Briefly, bone marrow was isolated, and fibroblasts and mature macrophages were removed by selective adhesion. The isolated monocytes were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 20 mM HEPES (Mediatech Cellgro, Herndon, VA), 10% FBS (Life Technologies), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (BioWhittaker, Walkersville, MD), 1× L-glutamine (Mediatech Cellgro), and 20% L-Cell supernatant as a source of M-CSF. After 4 days in culture, BMM$\phi$ were supplied fresh medium, and mature macrophages were harvested on day 7 and frozen at −140°C. Thawed macrophages were cultured on non-tissue culture plates for 3–7 days, passaged, and allowed to recover for 3–6 days, and then replated at $\sim 3 \times 10^5$ cells/35-mm tissue culture plate. The cells were allowed to adhere for 24 h before infection.

For all experiments, mycobacteria were added to macrophages on ice and incubated for 10 min, allowing mycobacteria to settle onto the cells, and then incubated at 37°C in 5% $CO_2$ for the specified times. Culture medium without antibiotics or L-cell supernatant was used in place of complete medium during the infections. For the 9-h time points, the BMM$\phi$s were incubated for 4 h with the mycobacteria and DMSO or inhibitors, washed with PBS three times, and then 2 ml of fresh medium was added with or without inhibitors and incubated for an additional 5 h. All tissue culture reagents were found negative for endotoxin contamination using either the E-Toxate assay (Sigma-Aldrich, St. Louis, MO) or QCL-1000 Endotoxin test (Cambrex Bio Science, Walkersville, MD).

### *Inhibitor treatments*

The inhibitors were purchased from Calbiochem (La Jolla, CA), reconstituted in sterile, endotoxin-tested DMSO, and used under the following conditions: BAPTA-AM (30 $\mu$M) was added at the time of infection; W7 (25 $\mu$M), KN-62 (10 $\mu$M), and KN-93 (10 $\mu$M) were added 30 min before infection; and H89 (20 $\mu$M) and KT5720 (10 $\mu$M) were added 1 h before the infection. For the cAMP add-back experiments, 8-Br-cAMP (10 $\mu$M) was added to the macrophages 30 min after infection. DMSO was used in the same concentrations as the vehicle control. For all inhibitors, a dose response was observed in relation to ERK1/2 phosphorylation, and the concentrations used in subsequent studies were chosen based on the dose response and previous studies published with macrophages (6, 15, 16). None of the inhibitors used had a significant effect on the macrophage's uptake of the mycobacteria.

### *Bacteria culture*

To generate *M. avium* 724 stocks, the mycobacteria (generously provided by A. Cooper (Trudeau Institute, Saranac Lake, NY)) were passaged through a mouse to ensure virulence, and a single colony was used to inoculate Middlebrooks 7H9 medium (Difco, Sparks, MD) supplemented with glucose, oleic acid, albumin, Tween 20, and NaCl (GOATS). Bacteria were grown for 10 days at 37°C with vigorous shaking, resuspended in Middlebrooks/GOATS with 15% glycerol, aliquoted, and stored at −80°C. Frozen stocks were quantitated by serial dilution onto Middlebrooks 7H10 agar/GOATS. *M. smegmatis* strain $MC^2155$ (generously provided by R. Groger (Washington University, St. Louis, MO)) was grown in Middlebrooks/GOATS at 37°C for 2–4 days. Frozen stocks were prepared as described for *M. avium*. All reagents used to grow mycobacteria were found negative for endotoxin contamination using the E-Toxate assay (Sigma-Aldrich) and the QCL-1000 endotoxin test (Cambrex Bio Science).

### *Mycobacteria infection*

Infection assays evaluated by fluorescence microscopy were performed on each stock of mycobacteria to determine the infection ratio needed to obtain ~80% of the macrophages infected. Briefly, BMM$\phi$ were plated on glass coverslips and infected with different doses of mycobacteria in triplicate. Infections were halted at either 1 or 4 h and fixed in 1:1 methanol: acetone, washed with PBS, and stained with TB Auramine M Stain kit (BD Biosciences, Sparks, MD) in the case of *M. avium*, and with acridine orange (Sigma-Aldrich) in the case of *M. smegmatis*. Slides were visualized using fluorescent microscopy, and the level of infection was quantitated by counting the number of cells infected in at least four fields per replicate. No fewer than 100 cells per replicate were counted.

### *Complement opsonization*

Appropriate concentrations of mycobacteria were suspended in macrophage culture medium containing 10% normal human serum (NHS) as a source of complement components and incubated for 2 h at 37°C (17). The NHS came from the same donor for all experiments. The same concentration of NHS was added to uninfected controls for all experiments.

### *CaMK activity assay*

After infection with mycobacteria, the BMM$\phi$ were lysed with ice-cold lysis buffer as described below; cell lysates were removed and used for the CaMKII kinase assay using the CaMK Kinase II Assay kit (Upstate, Lake Placid, NY) in the presence of 1 mM EGTA. The kinase reaction mixture contained 5 mM MOPS (pH 7.2), 5 mM $\beta$-glyceraldehyde phosphate, 1 mM EGTA, 0.2 mM DTT, 100 $\mu$M Autocamtide 2, 8 $\mu$g/ml CaM, PKA, PKC inhibitor mixture (provided with the kit), 15 mM $MgCl_2$, 100 $\mu$M ATP, and 5 $\mu$Ci (3000 Ci/mmol) of [$\gamma$-$^{32}$P]ATP. Kinase reactions were initiated by the addition of freshly prepared cell lysates to the reaction mixture at 30°C. After 30 min, the reaction was terminated by spotting onto phosphocellulose paper (Whatman, Clifton, NJ). The paper was washed three times with 0.75% phosphoric acid and finally with acetone. The [$\gamma$-$^{32}$P]ATP incorporation was measured using a scintillation counter (Beckman Coulter, Fullerton, CA).

### *Western blot analysis*

At designated times, the treated BMM$\phi$s were removed from the incubator and placed on ice. The culture medium was collected and saved for subsequent ELISAs, and the cells were washed three times with ice-cold PBS containing 1 mM pervanadate. The cells were then treated for 5–10 min with ice-cold lysis buffer (150 mM NaCl, 1 mM PMSF, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin, 1 mM pervanadate, 1 mM EDTA, 1% Igepal, 0.25% deoxycholic acid, 1 mM NaF, and 50 mM Tris-HCl (pH

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7.4)). The cell lysates were removed from the plates and stored at −20°C. Equal amounts of protein, as defined using the Micro BCA Protein Assay (Pierce, Rockford, IL), were loaded onto 10% SDS-PAGE gels, electrophoresed, and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked in TBS with 0.05% Tween 20 (TBST) supplemented with 5% powdered milk and then incubated with primary Abs against phospho-p38, total p38, phospho-ERK1/2, or total ERK1/2 from Cell Signaling (Beverly, MA). The blots were washed with TBST and incubated with a secondary Ab, either HRP-conjugated anti-rabbit or anti-mouse Ig (Pierce) in TBST plus 5% powdered milk. The bound Abs were detected using SuperSignal West Femto enhanced chemiluminescence reagents (Pierce). Densitometry was performed on some blots using the LKB Bromma Ultroscan XL Enhanced Laser Densitometer with GelScan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden).

### *ELISA*

The levels of TNF-$\alpha$ secreted into the culture medium by infected macrophages were measured using the BD PharMingen (San Diego, CA) OptEia Mouse TNF-$\alpha$ ELISA kit. Culture medium collected from the macrophages was analyzed for cytokines according to manufacturer's instructions, and the cytokine concentrations were determined against TNF-$\alpha$ standard curves. For the intracellular cAMP determinations, macrophages were seeded on 24-well plates and infected with mycobacteria as described above. Experiments were stopped by the addition of lysis buffer supplied in the cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences, Piscataway, NJ) and used in subsequent ELISAs according to the manufacturer's instructions.

### *Statistical analysis*

Statistical significance was determined with the paired two-tailed Student *t* test at $p < 0.05$ level of significance, using InStat/Prism software.

## Results

### *Macrophages infected with* M. smegmatis *maintain prolonged activation of p38 and ERK1/2 and increased TNF-α production compared with* M. avium *724-infected cells*

In previous studies, we found that an 80% infection level in BMM$\phi$, infected with either *M. smegmatis* or *M. avium* 724, showed significant differences in MAPK activation and TNF-$\alpha$ production (6). In Fig. 1, we confirmed these previous results and demonstrate that macrophages infected with *M. smegmatis* show prolonged p38 and ERK1/2 activation (Fig. 1, *A* and *B*) and significantly more TNF-$\alpha$ production (*C*) at each level of macrophage infection compared with *M. avium* 724. The number of mycobacteria needed to obtain a given percentage of infected macrophages was determined in preliminary experiments as described in *Materials and Methods*. For all of the infection doses, we observed ~1–10 mycobacteria per macrophage.

### *$Ca^{2+}$ is required for ERK1/2 activation in macrophages following mycobacterial infection*

It has recently been demonstrated that macrophages that phagocytose dead *Mycobacterium tuberculosis* have a transient rise in intracellular $Ca^{2+}$, and that this $Ca^{2+}$ release is important in initiating the phagosome maturation process (18, 19). Interestingly, phagocytosis of live *M. tuberculosis* did not induce a $Ca^{2+}$ flux, and as expected, the *M. tuberculosis* phagosome did not mature to a phagolysosome. Furthermore, $Ca^{2+}$/CaM has recently been shown to be necessary for the production and deposition of phosphatidylinositol 3-phosphate onto phagosomes, a process that is

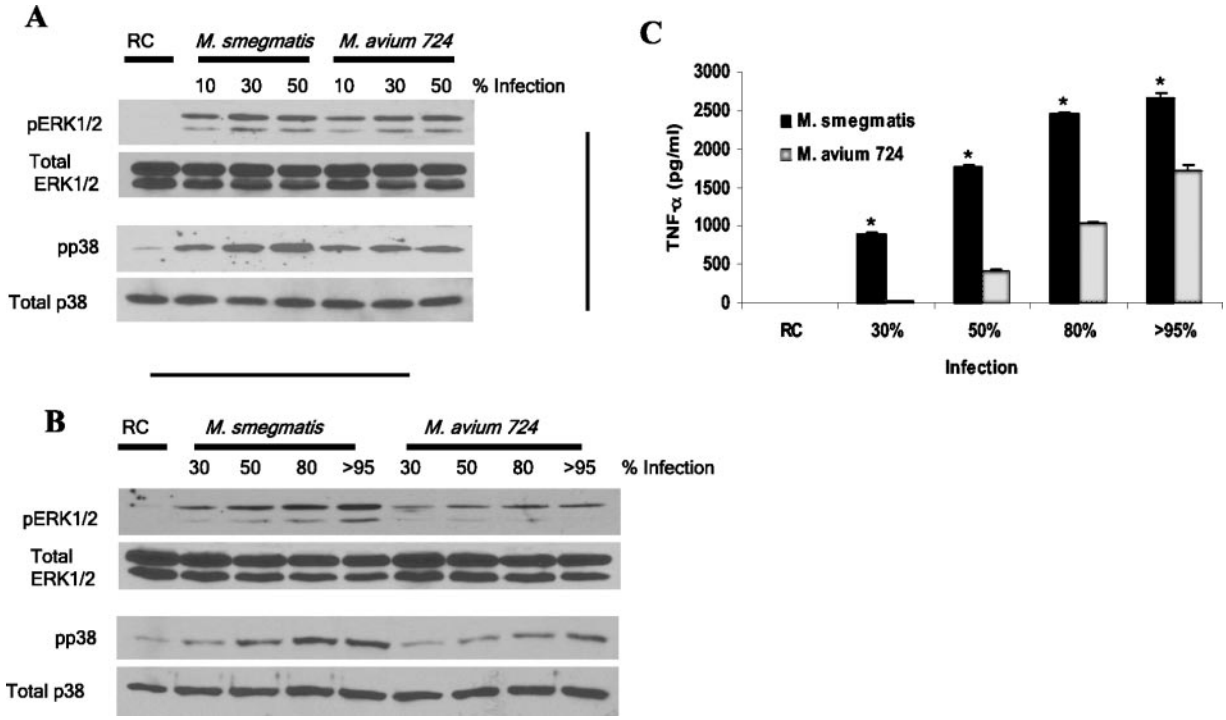


**FIGURE 1.** Infection with nonpathogenic *M. smegmatis* compared with pathogenic *M. avium* 724 induces prolonged activation of MAPKs and increased TNF-$\alpha$ production in macrophages in a dose-dependent manner. BMM$\phi$ were infected with *M. smegmatis* and *M. avium* 724 for 1 h to get 10, 30, and 50% ingestion of mycobacteria. For 9-h infection, BMM$\phi$ were infected with mycobacteria for 4 h to get 30, 50, 80, and >95% ingestion, and then washed, and fresh medium was added to the cells and the infection was continued for an additional 5 h. Shown are Western blots of BMM$\phi$ cell lysates probed for activated ERK1/2 and p38 after infection with *M. smegmatis* or *M. avium* 724 for 1 h (*A*) and 9 h (*B*). Total ERK1/2 and p38 blots were run to show equal protein loading. For TNF-$\alpha$ production, culture supernatants after 9 h of infection were analyzed by ELISA (*C*). ∗, *M. smegmatis* is significant to *M. avium* 724 at $p < 0.001$ by two-tailed Student's *t* test. Values are expressed as mean ± SD. Data are representative of three separate experiments.

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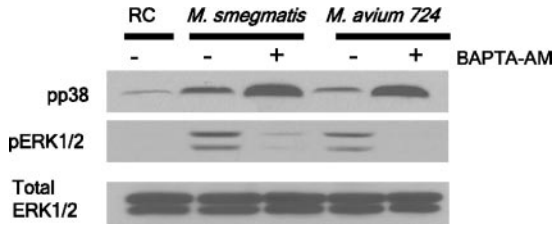


**FIGURE 2.** ERK1/2 activation but not p38 activation requires intracellular $Ca^{2+}$ upon mycobacterial infection. Shown are the Western blots of lysates probed for activated ERK1/2 and p38 after infection with *M. smegmatis* or *M. avium* 724 in BMM$\phi$. Macrophages were treated with BAPTA-AM or DMSO, as a vehicle control (−), at the time of infection. One-hour postinfection, the cell lysates were analyzed by Western blotting using phospho-specific Abs to ERK1/2 and p38 as described in *Materials and Methods*. Blots were then probed with total ERK1/2 Ab to show equal protein loading. These results are representative of three separate experiments.

inhibited in lipoarabinomannan (LAM)-treated macrophages (20). These and other papers suggest that limiting a macrophage $Ca^{2+}$ response may be an important feature of mycobacterial pathogenesis. To address whether $Ca^{2+}$ signaling was important in other aspects of a mycobacterial infection and whether its role in signaling differs between macrophages infected with pathogenic and nonpathogenic mycobacteria, we infected BMM$\phi$ with pathogenic *M. avium* 724 and nonpathogenic *M. smegmatis* in the presence or absence of the $Ca^{2+}$ chelator BAPTA-AM. We were particularly interested in whether $Ca^{2+}$ functioned upstream of the MAPK, because our previous studies indicated an important role for this family of kinases in macrophage activation following mycobacterial infection (6). We looked at 1 h postinfection, because our previous work showed that the MAPKs are highly activated in both *M. avium* 724 and *M. smegmatis*-infected macrophages. In BMM$\phi$ treated with BAPTA/AM and infected with *M. avium* or *M. smegmatis*, we observed a complete inhibition of ERK1/2 phosphorylation at 1 h postinfection as shown in Fig. 2. The importance of $Ca^{2+}$ in MAPK activation was specific to ERK1/2, because blocking $Ca^{2+}$ did not inhibit p38 phosphorylation. In contrast, we observed an increase in p38 activation in BAPTA-AM-treated BMM$\phi$, suggesting some role for $Ca^{2+}$ or a $Ca^{2+}$-dependent protein in down-regulating the p38 pathway.

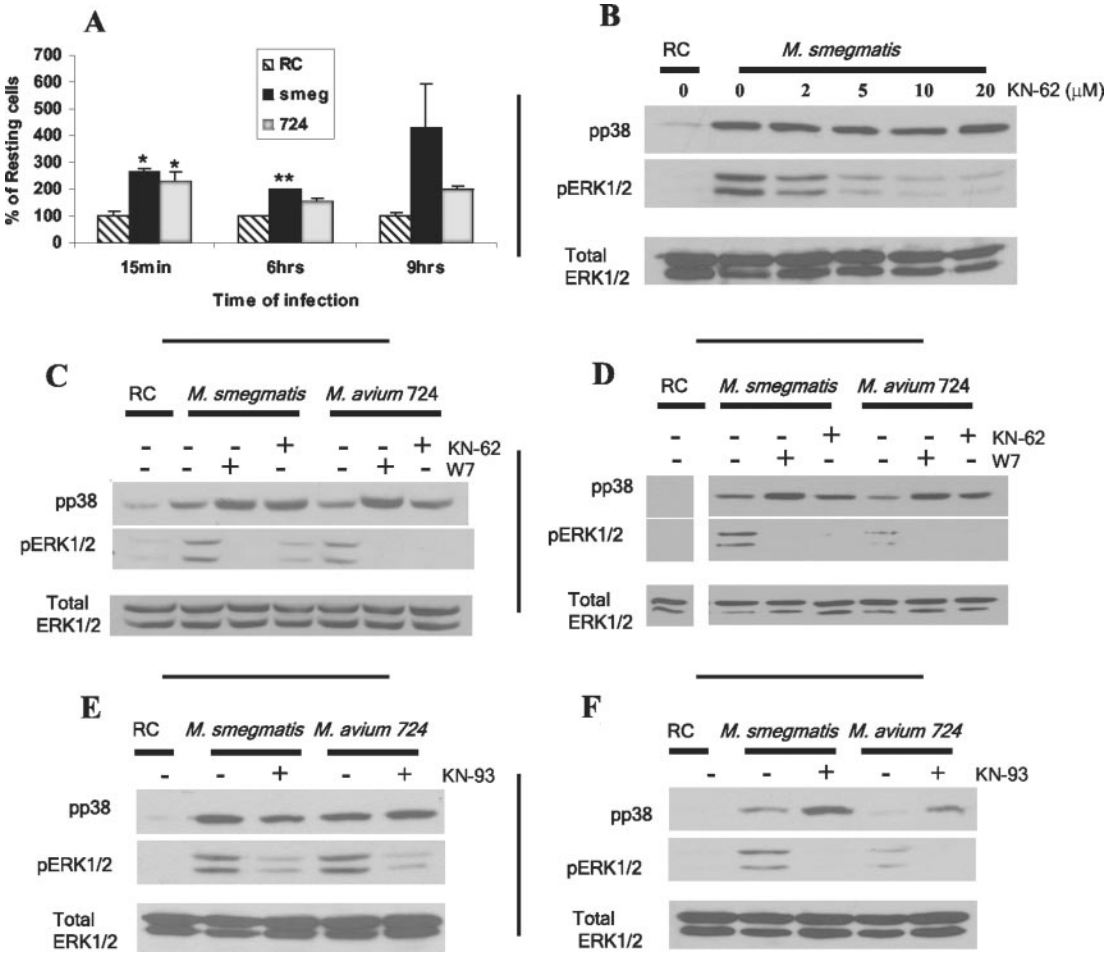


**FIGURE 3.** CaMKII is activated in macrophages infected with mycobacteria and is required for early and sustained activation of ERK1/2 in mycobacterial-infected BMM$\phi$. *A*, BMM$\phi$ were infected with *M. smegmatis* or *M. avium* 724 for 15 min, 6 h, and 9 h as described in *Materials and Methods*. Cell lysates were removed and screened for CaMKII activity. Values are expressed as mean ± SD. *, Significant to RC. **, Significant to RC and *M. avium* 724 ($p < 0.05$). Similar results were observed in three separate experiments. *B*, Dose-dependent inhibition of ERK1/2 was seen with varying concentrations of KN-62 in BMM$\phi$ infected with *M. smegmatis* for 1 h. *C–E*, BMM$\phi$ were treated with W-7, KN-62, KN-93, or DMSO (−) as vehicle control, for 30 min before the infection with *M. smegmatis* or *M. avium* 724 for 1 h (*C* and *E*) and 9 h (*D* and *F*) and lysed after the indicated times. For the 9-h infection, cells were infected with mycobacteria for 4 h and washed, and fresh medium containing DMSO or inhibitors was added, and the cells were harvested after an additional 5 h. BMM$\phi$ cell lysates were probed for activated ERK1/2 and p38. Total ERK1/2 blots were run to show equal protein loading. Results are representative of three separate experiments.

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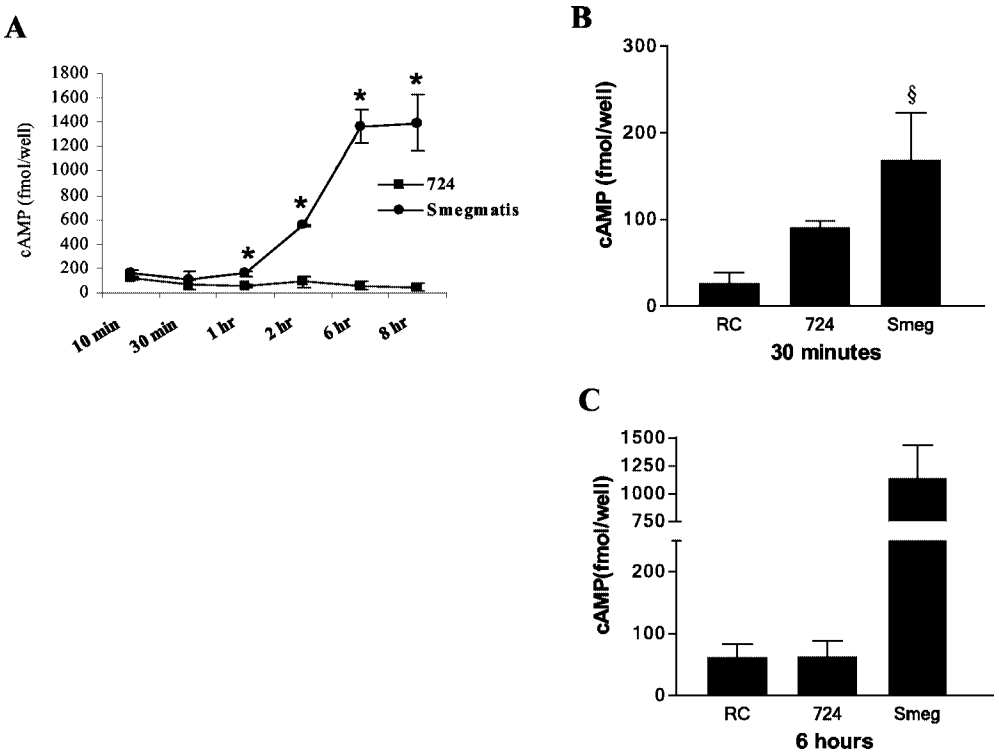


**FIGURE 4.** *M. smegmatis*-infected BMM$\phi$ produce significantly higher levels of cAMP as compared with *M. avium*-infected BMM$\phi$. Macrophages were infected with *M. avium* 724 or *M. smegmatis* for 10 min, 30 min, 1 h, 2 h, 6 h, or 8 h. Cell lysates were prepared for each time point and used for subsequent cAMP ELISA (*A*). cAMP production in BMM$\phi$ after a 30-min infection (*B*) and a 6-h infection (*C*). *, $p < 0.05$ by two-tailed Student's *t* test. §, *M. smegmatis* is significant to RC at $p < 0.05$ by two-tailed Student's *t* test. Values are expressed as mean ± SD. These data are representative of three separate experiments.

### *CaM and CaMK are required for ERK1/2 activation*

To define the mechanism by which alterations in $Ca^{2+}$ signaling are coupled to downstream ERK1/2 signaling, we looked at the possible involvement of CaM and CaMK. CaM is a calcium-sensing protein that undergoes a conformation change upon binding calcium and is an important cofactor for a number of enzymes including inducible NO synthase (21). CaM also functions in activating gene transcription, and this is mediated, at least in part, by the activation of CaMKs. These CaMKs can directly phosphorylate transcription factors or work through activation of other kinases including the MAPKs (14). To investigate the kinetics of CaMKII in mycobacterial infection, we measured the $Ca^{2+}$-independent activity of CaMKII in macrophages infected with *M. smegmatis* or *M. avium* 724 (Fig. 3*A*). CaMKII is activated at elevated levels in macrophages following mycobacterial infection as compared with resting cells (RC) at 15 min, 6 h, and 9 h. However, after an initial activation (i.e., 15 min), CaMKII is more active at 6 and 9 h postinfection in macrophages infected with *M. smegmatis* as compared with *M. avium*-infected cells.

To define the role of CaM/CaMK in MAPK activation, we treated BMM$\phi$ with W7 (a specific inhibitor of CaM) and KN-62 and KN-93 (inhibitors of CaMKII). Fig. 3*B* shows that ERK1/2 activation in macrophages following infection with *M. smegmatis* was strongly inhibited by CaMK inhibitor KN-62 in a dose-dependent manner. Based on this, we used 10 $\mu$M concentration of KN-62 for all the later experiments. Similar dose-response experiments were done with another CaMK inhibitor, KN-93, to determine the appropriate concentration (data not shown). As shown in Fig. 3, *C* and *E*, the addition of W7, KN-62, and KN-93 resulted in almost a complete loss of ERK1/2 phosphorylation following a 1-h *M. smegmatis* or *M. avium* infection. Because the cAMP response following a mycobacterial infection is maximum at 6–8 h postinfection (see below), we looked at MAPK activation after 9 h in both the CaM/CaMK and cAMP/PKA experiments. We observed a similar loss of ERK1/2 activation in macrophages infected for 9 h with *M. smegmatis* following inhibitor treatment (Fig. 3, *D* and *F*). As observed previously (6), infection of BMM$\phi$ with *M. avium* 724 fails to maintain a significant level of ERK1/2 activation by 4 h postinfection. Therefore, the effect of these inhibitors on ERK1/2 phosphorylation was minimal at this 9-h time point in *M. avium* 724-infected BMM$\phi$. p38 activation remained unaffected or increased after inhibition of CaM or CaMK in both *M. smegmatis*- and *M. avium*-infected BMM$\phi$ at 1 and 9 h postinfection. These data implicate an important role for CaM and CaMK in mediating ERK1/2 activation following mycobacterial infections.

### *cAMP production is significantly elevated in* M. smegmatis *compared with* M. avium *724-infected macrophages*

Numerous upstream signaling pathways converge on the MAPKs and, through their activation, induce a wide range of cellular effects including growth and differentiation, stress responses, and inflammatory responses. Therefore, we looked for other signaling molecules in addition to CaMKII that may be upstream of MAPK activation following mycobacterial infections and whether these signaling pathways were differentially regulated by *M. avium* compared with *M. smegmatis*. We focused our studies on the cAMP/PKA pathway, because this pathway has been shown to regulate a number of downstream effectors in macrophages including the MAPKs (8, 22) and CREB (23) and has been shown to either stimulate or inhibit inducible NO synthase and TNF-$\alpha$ production (13). To evaluate the role of cAMP/PKA, we measured cAMP

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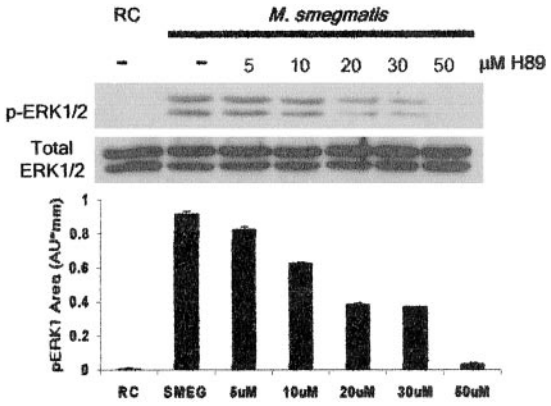


**FIGURE 5.** Dose response of ERK1/2 activation in BMM$\phi$ infected with *M. smegmatis* for 1 h following H89 treatment. Shown are Western blots of BMM$\phi$ cell lysates probed for activated ERK1/2 after infection with *M. smegmatis*. Macrophages were treated with 5, 10, 20, 30, and 50 $\mu$M H89 or DMSO vehicle control (−) for 1 h before a 1-h infection with *M. smegmatis*. The relative densities of the upper ERK1/2 band were analyzed by densitometry. AU, Arbitrary units. The results are representative of two separate experiments.

levels following *M. avium* 724 and *M. smegmatis* infections. Macrophages were infected to an equivalent extent with either *M. smegmatis* or *M. avium* 724 for 10 min, 30 min, 1 h, 2 h, 6 h, and 8 h, and the cell lysates were collected for analysis of intracellular cAMP production. As shown in Fig. 4*A*, macrophages infected with *M. smegmatis* induced a highly significant increase in cAMP production compared with *M. avium* 724 infection, and high levels of cAMP were maintained even 8 h post-*M. smegmatis* infection. At 30 min, there was significantly more cAMP in *M. smegmatis*-infected macrophages compared with uninfected RC and approximately twice as much as in *M. avium* 724-infected cells (Fig. 4*B*). *M. avium* 724-infected macrophages also showed a 2-fold increase in cAMP production compared with RC at 30 min (Fig. 4*B*); however, this small increase was only transient, because by 6 h, *M. avium* 724 had RC levels of cAMP production (Fig. 4*C*).

### *PKA is necessary for ERK1/2 activation in macrophages following mycobacterial infection*

Because the cAMP production data correlated with the sustained ERK1/2 activation observed in *M. smegmatis*-infected macrophages, we tested whether PKA activity was required for ERK1/2 phosphorylation. We tested varying concentrations of the PKA-specific inhibitor H89 on ERK1/2 activation and saw a dose response following an *M. smegmatis* infection (Fig. 5). For the remaining experiments, a 20 $\mu$M concentration of the inhibitor was used, which is consistent with previously published protocols using murine macrophages (15). As shown in Fig. 6*A*, pretreating the macrophages with the PKA inhibitor H89 resulted in a significant reduction in ERK1/2 activation following a 1-h *M. smegmatis* or *M. avium* 724 infection. The prolonged activation of ERK1/2 in *M. smegmatis*-infected macrophages was also dependent on PKA, because H89 completely blocked ERK1/2 phosphorylation, even 9 h postinfection (Fig. 6*B*). Because *M. avium* 724-infected macrophages showed only slight ERK1/2 activation at this later time point, the addition of H89 had little effect. However, adding the membrane-permeable cAMP analog 8-Br-cAMP to *M. avium* 724-infected macrophages caused increased ERK1/2 activation (data not shown).

To confirm a role for PKA in ERK1/2 activation, we also treated BMM$\phi$ with KT5720, another PKA specific inhibitor. As shown in Fig. 6*C*, KT5720 had the same effect on ERK1/2 activation as the PKA inhibitor H89. These data suggest that the continued activation of cAMP/PKA by macrophages infected with *M. smegmatis* is required for the sustained ERK1/2 activation, a characteristic of these infected cells.

### *cAMP production in macrophages following mycobacterial infection is CaM and CaMK dependent*

As shown in Figs. 3 and 6, regulation of ERK1/2 following mycobacterial infection is dependent on both the CaM/CaMK and cAMP/PKA pathways. To determine whether the CaM/CaMK pathway is upstream of the cAMP/PKA pathway, or is simply a separate pathway that converges on ERK1/2, we used the inhibitors W7 and KN-62 and evaluated cAMP production. We treated macrophages with the inhibitors 30 min before infection with *M. smegmatis* and measured cAMP production at 30 min, 6 h, and 8 h postinfection. Fig. 7 shows that there is a significant decrease in cAMP levels following treatment with either KN-62 or W7 in macrophages infected with *M. smegmatis*. Macrophages were also infected with *M. avium* 724 and treated with the inhibitors; however, the limited and transient cAMP response observed in *M. avium* 724-infected BMM$\phi$ was not blocked by either inhibitor (data not shown).

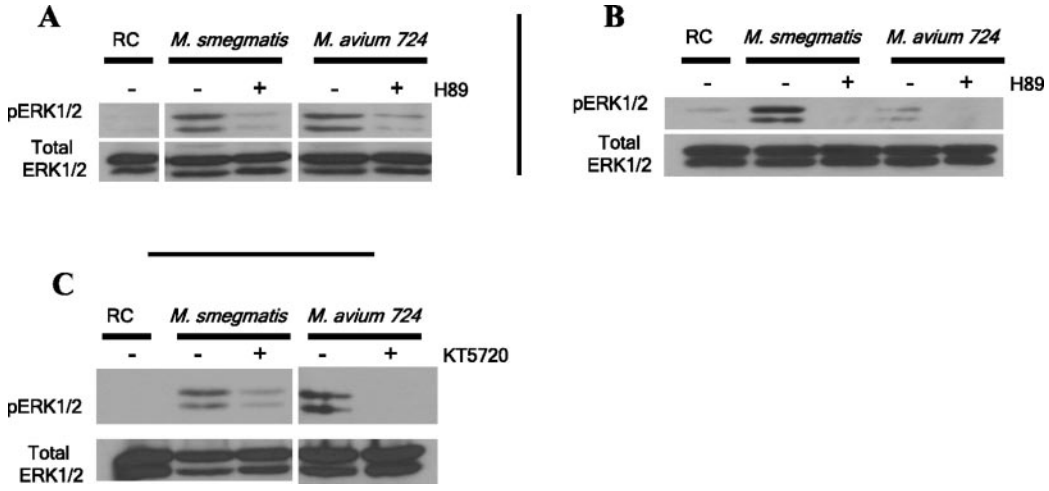


**FIGURE 6.** ERK1/2 activation at 1- and 9-h postinfection requires PKA activity in both *M. smegmatis*- and *M. avium*-infected BMM$\phi$. Shown are Western blots of BMM$\phi$ cell lysates probed for activated ERK1/2 after infection with *M. smegmatis* or *M. avium* 724 for 1 h (*A*) and 9 h (*B*) and for 1 h in the KT5720 experiment (*C*). Macrophages were treated with H89, KT5720, or DMSO (−), 1 h before infection and lysed after indicated times. For the 9-h infection, cells were treated the same way as described in Fig. 3.

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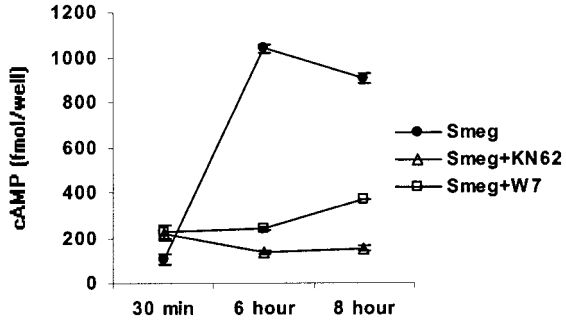


**FIGURE 7.** Increased cAMP activation in BMM$\phi$ following *M. smegmatis* infection is dependent on the CaM/CaMK pathway. BMM$\phi$ were treated with KN-62 or W7 or with DMSO, as a vehicle control, for 30 min before infection as described in Fig. 3. Macrophages were infected with *M. smegmatis* or *M. avium* 724 and harvested at the indicated times, and cell lysates were analyzed for cAMP concentration by ELISA. Values are expressed as mean ± SD. These data are representative of three separate experiments.

These data demonstrate that the cAMP production in *M. smegmatis*-infected macrophages is mediated by the CaM/CaMK pathway at infection times where high cAMP expression is observed.

### *TNF-α production is dependent on the CaM/CaMK and cAMP/PKA pathways in* M. smegmatis-*, but not* M. avium-*infected BMMϕs*

Both the $Ca^{2+}$/CaM/CaMK and cAMP/PKA pathways have been implicated in signal transduction cascades leading to production of inflammatory cytokines such as TNF-α (10, 24) and IL-8 (25). However, as discussed above, the cAMP/PKA pathway has also been shown to inhibit TNF-α production (26) and IL-12 production (27). Additionally, we and others (4, 6, 28, 29) have shown that macrophages infected with fast-growing, nonpathogenic mycobacteria produce significantly higher TNF-α levels compared with macrophages infected with pathogenic *M. avium*, and this response was dependent on the MAPKs. Therefore, we investigated whether activation of the $Ca^{2+}$/CaM/CaMK and cAMP/PKA pathways was required for TNF-α production in macrophages following an *M. smegmatis* infection. We found that the TNF-α production elicited by an *M. smegmatis* infection was inhibited by W7, KN-62 (Fig. 8*A*), and H89 (*B*). However, the level of TNF-α production was unchanged after inhibition of CaM/CaMK and decreased only slightly after inhibition of cAMP/PKA in *M. avium* 724-infected macrophages, suggesting that *M. smegmatis* uses the $Ca^{2+}$/CaM/CaMK and cAMP/PKA pathways to enhance the TNF-α response, whereas *M. avium*'s comparatively low production is independent of these pathways. Similar results were seen with KN-93, which inhibited TNF-α production in *M. smegmatis*-infected macrophages and did not affect TNF-α produced in macrophages infected with *M. avium* 724 (data not shown). No TNF-α production was detected in RC (data not shown). These results indicate a differential induction of CaM/CaMK and cAMP/PKA pathways by *M. smegmatis* and *M. avium* 724, which is responsible for the disparity in ERK1/2 activation and TNF-α production. At early times following *M. avium* and *M. smegmatis* infection (i.e., 1 h), both CaM/CaMK and cAMP/PKA pathways are important for ERK1/2 activation. However, only in *M. smegmatis*-infected cells are increased levels of cAMP/PKA maintained for an extended period (i.e., 9 h), leading to prolonged ERK1/2 phosphorylation and increased TNF-α production. A summary of these results is depicted in Fig. 9.

## Discussion

Most virulent mycobacterial species are intracellular pathogens that reside safely within host macrophage phagosomes. Following infection, pathogenic mycobacteria interfere with the normal phagosome maturation process and block the formation of a phagolysosome. Moreover, macrophages infected with pathogenic mycobacteria induce a minimal Th1 cytokine response compared with their response to nonpathogenic species, and this likely also plays a role in enhancing the mycobacterial survival in vivo. Although a large body of literature has been devoted to defining the interaction between mycobacteria and the macrophage's phagosome, very little is known about the signaling events initiated in the macrophage following attachment and ingestion of mycobacteria. These signaling events are critical in the establishment of a safe environment for the bacterium within the macrophage and in the suppression of a generalized inflammatory response. Therefore, it is important to understand the signaling pathways activated in macrophages upon mycobacterial infection and whether pathogenic mycobacteria modulate these pathways as a virulence mechanism.

We have recently reported that virulent strains of *M. avium* differentially regulate the MAPK cascade relative to fast-growing, nonpathogenic mycobacteria such as *Mycobacterium phlei* and *M. smegmatis* (6). We showed that activation of p38 and ERK1/2 was sustained for long periods in macrophages infected with nonpathogenic mycobacteria relative to *M. avium* infections, and activation of ERK1/2 and p38 was necessary for subsequent TNF-α production. Other groups have reported differences in MAPK signaling in both human and murine macrophages following infection with different strains and morphotypes of *M. avium*, suggesting that activation of the MAPKs is an important antimycobacterial event in macrophages (30).

There are multiple upstream signaling pathways that have been shown to converge on ERK1/2 and p38 MAPKs in macrophages. However, only $PGE_2$ has been linked to regulating MAPK activity following a mycobacterial infection (31). We hypothesized that the $Ca^{2+}$/CaM/CaMK pathway could be involved in the macrophage signaling response based on the recent findings showing that activation of CaM/CaMK is required for the phagosome-lysosome fusion in macrophages infected with *M. tuberculosis* (18). Moreover, in a study by Vergne et al. (20), it was shown that Man-LAM

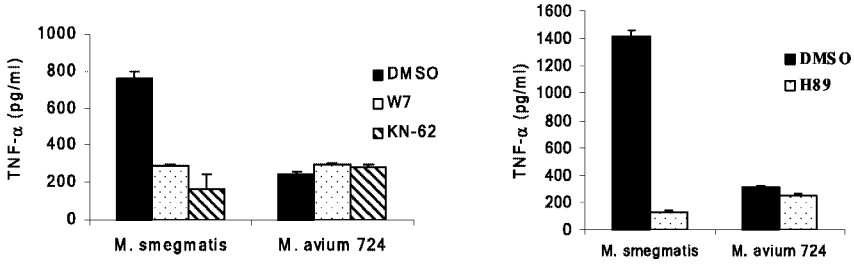


**FIGURE 8.** TNF-α production in BMM$\phi$ is dependent on the CaM/CaMK and cAMP/PKA pathways following an *M. smegmatis* but not *M. avium* 724 infection. BMM$\phi$ cells were treated with KN-62, W7, or DMSO for 30 min (*A*) and with H89 or DMSO for 1 h (*B*), before the addition of mycobacteria. Infection was continued for a total of 9 h, as described in Fig. 3. Culture supernatants were analyzed for TNF-α by ELISA. Values are expressed as mean ± SD. The results are representative of three separate experiments.

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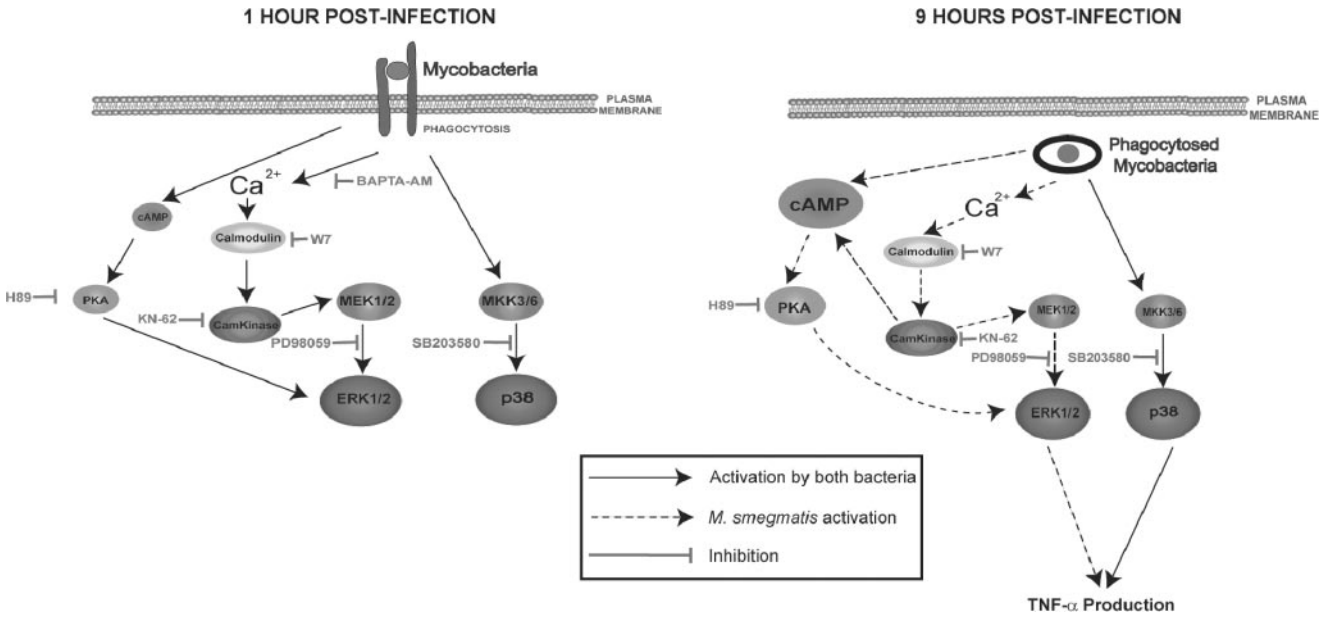


**FIGURE 9.** Conceptual model for the signal transduction cascades initiated in primary BALB/c BMM$\phi$s following infection by *M. smegmatis* and *M. avium* 724. *A*, Following a 1-h infection with *M. smegmatis* or *M. avium* 724, the ERK1/2 and p38 MAPK pathways are activated. The cAMP/PKA and CaM/CaMK pathways both activate ERK1/2 at this early time point; however, p38 activation was not dependent on these pathways. At 9 h postinfection, there are significant differences in the activation of these signaling molecules in *M. smegmatis*-infected cells compared with *M. avium* 724-infected cells. *M. smegmatis* infection in BMM$\phi$ induces a dramatic increase in cAMP/PKA activation (as denoted by the increased size of the cAMP representative oval), which is absent following an *M. avium* 724 infection. Additionally, the relative level of ERK1/2 and p38 activation, is dramatically reduced or even absent in *M. avium* 724-infected cells. TNF-$\alpha$ production in *M. smegmatis*-infected cells is dependent on both the cAMP/PKA and $Ca^{2+}$/CaM/CaMK pathways, whereas the limited TNF-$\alpha$ production in *M. avium* 724-infected BMM$\phi$ is independent of cAMP/PKA and CaM/CaMK.

from virulent *M. tuberculosis*, but not Ara-LAM from *M. smegmatis* blocked an A23187-induced increase in cytosolic $Ca^{2+}$. Therefore, we investigated the importance of cytosolic $Ca^{2+}$ in inducing various signaling pathways following *M. avium* 724 or *M. smegmatis* infection. In BMM$\phi$s pretreated with the intracellular $Ca^{2+}$ chelator BAPTA-AM, we observed a minimal activation of ERK1/2 following mycobacterial infections. However, p38 activation was slightly increased, highlighting a differential role played by $Ca^{2+}$ in the MAPK signal transduction cascade following the mycobacterial infections.

CaM is the most abundant and well-known $Ca^{2+}$ binding protein that regulates numerous $Ca^{2+}$-mediated/dependent cellular functions such as cell growth, differentiation, proliferation, cell survival, and motility (32). CaM functions are mediated by phosphorylation of cellular proteins through the activation of a variety of protein kinases, including CaM-dependent protein kinases (CaMKI, -II, -III, -IV, and -V) (33), phosphorylase kinase (34), and myosin L chain kinase (35). The most intensely investigated member of this multifunctional group is CaMKII. CaMKII is a serine/threonine kinase, which upon activation by $Ca^{2+}$/CaM binding, undergoes an immediate autophosphorylation on $Thr^{286}$ (36), thus relieving its autoinhibition. Hence, a transient elevation in $Ca^{2+}$ can lead to a prolonged activation of CaMKII. In the present study, we observed that, following a mycobacterial infection, there is an initial activation of CaMKII that was present in both *M. smegmatis*- and *M. avium* 724-infected macrophages. However, at later times (6 and 9 h), CaMKII is activated at higher levels in *M. smegmatis* relative to *M. avium* 724-infected macrophages. These results indicate that, upon infection with nonpathogenic *M. smegmatis*, there is an elevated activation of CaMKII as compared with *M. avium* 724. Using the CaM- and CaMKII-specific inhibitors W7 and KN-93/KN-62, respectively, we showed that ERK1/2 activation in primary BMM$\phi$ following a mycobacterial infection was dependent on the $Ca^{2+}$/CaM/CaMK pathway, and, not surprisingly, inhibition of CaM/CaMK caused an increase in p38 activation, similar to what was seen after chelation of intracellular $Ca^{2+}$.

The inhibition of ERK1/2 and slight increase observed in p38 phosphorylation following treatment with W7 and KN-62, underscores the fact that, although ERK1/2 and p38 can be activated by the same external stimuli, the subsequent signaling pathways that activate their respective MAPK kinases can be quite distinct. With the use of complement-opsonized mycobacteria, we would predict that complement receptors are playing a major role in mediating mycobacterial attachment and ingestion; however, there are likely other receptors engaged by the mycobacteria. Engagement of specific receptors could induce the $Ca^{2+}$/CaM/CaMK pathway with subsequent ERK1/2 activation, whereas entirely different receptors could be responsible for p38 activation. In addition, cross talk between the MAPK pathways has been shown previously (37–39), where the inhibition of one kinase increases the activation state of the other. This could be through regulation of MAPK-specific phosphatases (40); however, the exact mechanism for this cross talk is presently unclear.

We next investigated the role of the cAMP/PKA pathway in mycobacterial regulation of the host response to infection, because previous studies have shown this pathway to be important in cytokine production (7) and MAPK activation in macrophages (22). cAMP and its principal target PKA have been the subject of copious amounts of research since its discovery in the 1960s, and its importance in cellular regulation is well defined. cAMP is produced following binding of ligands to G protein-coupled receptors. Stimulatory G proteins then activate AC, which is responsible for the conversion of ATP to the secondary messenger cAMP. PKA is a tetramer composed of two regulatory subunits (R) and two catalytic subunits (C). Upon cAMP binding, the subunits rapidly dissociate, and the C

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subunits are allowed to phosphorylate a wide variety of proteins including kinases, transcription factors, and phosphatases (11). PKA has been shown to directly activate the transcription factor known as CREB, which acts on numerous cytokine promoters through the cAMP response element (23). Additionally, cAMP/PKA has been shown to both activate and suppress numerous inflammatory cytokines; however, its role in macrophage signaling upon a mycobacterial infection was unknown. We show a dramatic and highly significant activation of cAMP production in *M. smegmatis*-infected macrophages compared with *M. avium* 724-infected cells. Interestingly, studies using *Brucella* (41) and *Ehrlichia* (42) have reported that the increased cAMP production associated with the infected host cells is a virulence mechanism for these pathogens. In contrast, we observe a lack of or a suppression of cAMP production in *M. avium*-infected macrophages. However, in the *Brucella* and *Ehrlichia* studies, the levels of cAMP production were vastly lower than what we observed following an *M. smegmatis* infection. Indeed, they were near the levels we show for *M. avium*-infected BMM$\phi$. These observations suggest that increased activation of certain molecules downstream of cAMP, via an abundance of available cAMP, might tip the balance between a controlled infection and the lack thereof.

To begin testing this possibility, we used the PKA-specific inhibitors H89 and KT5720 to evaluate PKA's role in MAPK activation following mycobacterial infection. PKA is necessary for ERK1/2 activation 1 h postinfection in both *M. smegmatis*- and *M. avium*-infected cells. At this early time, both bacteria induce cAMP activation, although *M. smegmatis* is more efficient at activating cAMP than *M. avium*. However, at 8 h, *M. avium*-infected cells have almost undetectable amounts of cAMP, whereas *M. smegmatis*-infected cells have markedly elevated levels. In addition, at 9 h, we detect high levels of ERK1/2 activation in *M. smegmatis*-infected BMM$\phi$ that is PKA dependent.

We were also interested in the link between CaM/CaMK and ERK1/2 activation, because a number of pathways have been shown to be affected by CaM/CaMK for regulation of MAPK (43). A role for CaM in ERK1/2 activation has also been defined in fibroblasts where CaM was shown to bind to some isoforms of Ras for the regulation of ERK1/2 pathway (44, 45). $Ca^{2+}$/CaM-sensitive tyrosine kinase has also been shown to activate ERK1/2 pathway, leading to NF-$\kappa$B-mediated IL-8 production in *Helicobacter pylori* infection in macrophages (46). Because we observed that activation of ERK1/2 was dependent on the cAMP/PKA pathway, we hypothesized that CaM/CaMK could be regulating ERK1/2 activation through the cAMP/PKA pathway. In support of this hypothesis, some isoforms of AC can be activated by $Ca^{2+}$/CaM and CaMK (47). Additionally, in neutrophils, group 1 AC isoforms were shown to be activated upon addition of exogenous $Ca^{2+}$/CaM or stimulation with PMA, the activator of PKC (48). Other studies have shown regulation of AC isoforms by CaMKII and CaMKIV by phosphorylation of the serine residues on AC (49, 50). Indeed, inhibition of CaM and CaMK in our study with W7 and KN-62, respectively, resulted in diminished production of cAMP following *M. smegmatis* infection. This inhibition was particularly evident at 6 and 8 h postinfection. These data demonstrate that activation of CaM/CaMK is important for the increased cAMP production in *M. smegmatis*-infected BMM$\phi$ (depicted in Fig. 9). We hypothesize that CaMKII is regulating the activity of AC by direct phosphorylation; however, an indirect mechanism involving other signaling molecules cannot be excluded (49).

We have previously shown that the pronounced TNF-$\alpha$ production observed in macrophages following an infection with *M. smegmatis* is dependent on MAPK (6), and cytokine production in macrophages upon different stimuli has been linked to CaM/CaMK pathway (10, 24). West et al. (9) showed that, in LPS-treated macrophages, TNF-$\alpha$ production was dependent on $Ca^{2+}$/CaM, whereas IL-1 release was independent of $Ca^{2+}$-mediated signaling. To define whether TNF-$\alpha$ production upon mycobacterial infection requires activation of the CaM/CaMK and cAMP/PKA pathways, we looked at TNF-$\alpha$ production in mycobacterial-infected BMM$\phi$ pretreated with W7, KN-62, and H89. Inhibition of CaM/CaMK in *M. smegmatis*-infected BMM$\phi$ led to significantly diminished production of TNF-$\alpha$, and, because CaM/CaMK also mediates cAMP production, the same effect on TNF-$\alpha$ production was observed with H89 treatment. In contrast, in *M. avium* 724-infected BMM$\phi$, no effect on TNF-$\alpha$ production was seen after W7 or KN-62 treatment (Fig. 8*A*) or resulted only in a slight decrease after H89 treatment (*B*). These data support our hypothesis that increased cAMP production, which is dependent on CaM/CaMK activation, is responsible for the higher levels of TNF-$\alpha$ production observed in BMM$\phi$ infected with the nonpathogenic mycobacteria *M. smegmatis*.

Based on our findings, we propose a model for MAPK activation in BMM$\phi$s upon mycobacterial infection that includes events at the time of infection and hours postinfection, shown in Fig. 9. At early time points, both $Ca^{2+}$/CaM/CaMK and cAMP/PKA pathways are important for ERK1/2 activation upon infection with *M. smegmatis* and *M. avium*. However, *M. smegmatis*-infected cells maintain a higher level of cAMP at later time points compared with *M. avium*-infected cells, which results in enhanced production of TNF-$\alpha$ (Fig. 9).

At present, we do not understand the mechanism by which the mycobacteria induce such varied responses. With the use of complement-opsonized mycobacteria, we would predict that complement receptors are playing a major role in mediating mycobacterial attachment and ingestion; however, there are other receptors, such as mannose receptor, CD14, Toll-like receptor 2, or Toll-like receptor 4, that are also likely engaged by the mycobacteria. Engagement of particular receptors by mycobacteria could lead to the induction or suppression of a particular signaling pathway, resulting in modulation of the macrophage signaling response. Delineating the role of these various receptors in the immune response to mycobacterial infection using nonopsonized mycobacteria combined with the use of receptor knockout macrophages, would help define how mycobacteria modulate macrophage signal transduction.

It is also intriguing to speculate that some signaling complexes remain associated with the *M. smegmatis* phagosome, and perhaps new complexes are formed as the phagosome matures. In support of this possibility, recent studies by Kusner and colleagues (18) demonstrated that phagosomes containing dead *M. tuberculosis* have significantly elevated CaMK activity relative to phagosomes containing viable *M. tuberculosis*. Therefore, additional studies are needed to define the location of these signaling molecules in relation to the mycobacterial phagosome and whether they function to maintain the macrophages in an activated state.

In conclusion, our data demonstrate 1) $Ca^{2+}$ is important in ERK1/2 but not p38 activation following mycobacterial infection, and this is dependent on CaM and CaMK; 2) BMM$\phi$ infected with the nonpathogenic mycobacterium *M. smegmatis* show significantly higher cAMP production relative to cells infected with pathogenic *M. avium* 724; and 3) this increased cAMP production (dependent on CaM/CaMK pathway) is responsible for the differential levels of TNF-$\alpha$ induced by *M. smegmatis* and *M. avium*. This is the first report showing differential induction of cAMP by different species of mycobacteria, and that cAMP is an important activator of a macrophage proinflammatory response to a mycobacterial infection.

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