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Bacillus anthracis Spores Stimulate Cytokine and Chemokine Innate Immune Responses in Human Alveolar Macrophages through Multiple Mitogen-Activated Protein Kinase Pathways†

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Contact with the human alveolar macrophage plays a key role in the innate immune response to *Bacillus anthracis* spores. Because there is a significant delay between the initial contact of the spore with the host and clinical evidence of disease, there appears to be temporary containment of the pathogen by the innate immune system. Therefore, the early macrophage response to *Bacillus anthracis* exposure is important in understanding the pathogenesis of this disease. In this paper, we studied the initial events after exposure to spores, beginning with the rapid internalization of spores by the macrophages. Spore exposure rapidly activated the mitogen-activated protein kinase signaling pathways extracellular signal-regulated kinase, c-Jun-NH2-terminal kinase, and p38. This was followed by the transcriptional activation of cytokine and primarily monocyte chemokine genes as determined by RNase protection assays. Transcriptional induction is reflected at the translational level, as interleukin-1α (IL-1α), IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) cytokine protein levels were markedly elevated as determined by enzyme-linked immunosorbent assay. Induction of IL-6 and TNF-α, and, to a lesser extent, IL-1α and IL-1β, was partially inhibited by the blockade of individual mitogen-activated protein kinases, while the complete inhibition of cytokine induction was achieved when multiple signaling pathway inhibitors were used. Taken together, these data clearly show activation of the innate immune system in human alveolar macrophages by *Bacillus anthracis* spores. The data also show that multiple signaling pathways are involved in this cytokine response. This report is the first comprehensive examination of this process in primary human alveolar macrophages.

Anthrax, a virulent disease recognized since early human history, is caused by a gram-positive, aerobic, spore-forming, rod-shaped bacterium, *Bacillus anthracis*. Dormant endospores are highly resistant to environmental stresses such as heat, drying, and UV light (27). The three primary forms of the disease are due to three different mechanisms of exposure: ingestion (gastrointestinal), contact (cutaneous), and inhalation (inhalational) (7). Inhalational anthrax is considered to be the most life-threatening form of the disease (9, 10) and was the most lethal primary form identified during the recent bioterrorism attack in 2001 and with the accidental exposure during manufacture in Sverdlosk in 1979 (18).

Inhalational anthrax is characterized by a rather unique finding in that the inhaled spores do not vegetate and cause disease at the site of entry. Instead, spores are rapidly and efficiently phagocytosed by alveolar macrophages and are carried through lung tissue to the regional lymph nodes. During this process, the spores are able to survive, germinate into vegetative bacilli, multiply, and escape the control of the innate immune system (6, 13, 14). *B. anthracis* vegetative bacteria penetrate into the blood circulation by disrupting macrophages, and there is evidence that much of the tissue damage is caused through the action of three major virulence factors, capsule, edema toxin, and lethal toxin (LT) (24). Because the spores require ingestion and transport to the mediastinal lymph nodes by macrophages to cause disease, this cell has been the focus of studies as a potential "Trojan horse" used by the *Bacillus anthracis* spore to escape control by the innate immune system (12). Normally, alveolar macrophages play a central role in the innate immune system and are the first line of defense against inhaled pathogens. They are the most prominent resident cells that not only engulf and kill infectious agents but also produce numerous modulators of the inflammatory response to recruit and activate additional cells of the immune system. Alveolar macrophages also provide a link to the adaptive immune system since they function as antigen-presenting cells.

Thus, macrophages, though usually seen as sentinel cells in innate immunity, are also used by *Bacillus anthracis* spores to bypass host immune systems. Current studies of the interaction of *Bacillus anthracis* spores with macrophages use murine macrophage primary cells or cell lines (20) or differentiated human peripheral blood monocytes (3). For example, direct visual evidence of rapid spore internalization by monocyte/macrophage cell types has been shown only in mouse primary macrophages and in human peripheral blood monocytes differentiated to a dendritic cell phenotype (3, 12). In these studies,

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there is evidence for the induction of several cytokines including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α). The only chemokine studied, interleukin-8, is also induced.

Mitogen-activated protein kinases (MAPKs) are important regulators for cytokine gene expression. The three major MAPK pathways, extracellular signal-regulated kinase (ERK), c-Jun-NH2-terminal kinase (JNK), and p38 MAPK (22), are important for the induction of numerous cytokine mediators of the innate immune response. The role of MAPK activation in cytokine induction by *Bacillus anthracis* spores in monocyte-derived cells has not been definitively examined, although it has been shown to be coincident with the induction of the signaling components ERK1/2, p38, and, to a lesser extent, stress-activated protein kinase (SAPK)/JNK (3). Of additional interest, LT produced by vegetative *Bacillus anthracis* bacteria inhibits MAPK signaling through the cleavage of upstream MAPK kinase (19).

Our current study demonstrates the rapid internalization of *Bacillus anthracis* spores by primary human alveolar macrophages followed by the activation of the innate immune system as evidenced by the induction of cytokines and chemokines. The chemokines induced are primarily monocyte, but not neutrophil or lymphocyte, chemotaxins. We also demonstrate that the induction of the MAPK cascades involving ERK1/2, P38, and SAPK/JNK are causally related to cytokine and chemokine induction by using chemical inhibitors of these pathways. Our paper is the first to examine these issues in detail in primary human alveolar macrophages.

MATERIALS AND METHODS

Preparation of *Bacillus anthracis* spores. *Bacillus anthracis* Sterne strain 7702 (pX01$^+$ pX02$^-$) was kindly provided by Jimmy Ballard (University of Oklahoma Health Sciences Center, Oklahoma City, Okla.). Bacteria were grown overnight at 37°C with continuous shaking in LB medium and were then streaked onto Arret and Kirchbaum agar sporulating slants. Bacteria were incubated for 3 weeks at 30°C. The slants were washed with 10 ml of chilled, sterile, deionized water; spun at 10,000 × *g* for 10 min; and resuspended in 10 ml chilled water. The spore suspension was heated at 65°C for 30 min to kill vegetative bacteria. After heat treatment, the spores were centrifuged for 10 min at 10,000 × *g*. The pellet was washed five times to remove contaminating cell debris. The supernatant and the very top layer of the pellet were aspirated and discarded each time, and the spores were then resuspended in chilled sterile deionized water and centrifuged for 10 min. The titer of the spore preparation was determined by plate counts. The spores were diluted to 1×10^9 spores/ml and stored at 4°C. Titers were reconfirmed by plate counts before each use. There was no detectable endotoxin in the final spore dilutions used in the experiments as determined by a limulus amebocyte lysate assay (Cambrex, Walkersville, MD).

Collection of human alveolar macrophages. Macrophages were obtained by bronchoscopy with the signed informed consent of human subjects according to a protocol approved by the Oklahoma University Health Sciences Center Institutional Review Board and the Institutional Biosafety Committee. These volunteers were healthy nonsmoking subjects, aged 18 to 35 years, with no history of pulmonary or cardiac disease or recent infections. Cells from human subjects were collected in sterile saline solution and centrifuged at 500 × *g* for 5 min. The supernatant was removed, and the pellets were washed in 10 ml of RPMI 1640 medium containing 50 μg/μl gentamicin and resuspended in 10 ml RPMI medium plus 2% fetal calf serum (FCS) with 50 μg/ml gentamicin. Cell counts were determined by a hemocytometer, the cell type was determined by morphology using Diff-Quick staining (Baxter, Miami, FL), and cells were resuspended to a concentration of 1×10^6 macrophages/ml. There were >95% macrophages in each cell preparation. One milliliter of cells per well was plated onto 24-well plates and allowed to incubate for 2 to 4 h to facilitate attachment. Subsequently, the medium was removed, and fresh medium containing 2% FCS and gentamicin was added. The cells were incubated overnight at 37°C with 5% CO_2.

Determination of intracellular spore and bacterial counts. Spore internalization was performed as described previously by Pickering et al. (21), with modifications. Briefly, the human macrophages were infected with 1×10^6 spores/ml (multiplicity of infection [MOI] of 1). The infection was allowed to proceed for 15, 30, 60, or 120 min in RPMI medium containing 2% FCS without gentamicin. Following each incubation time, the supernatant was removed, and the cells were washed twice with phosphate-buffered saline (PBS) and then lysed with 0.05% deoxycholate. Cell-associated counts were made by plating onto LB agar plates. Half the cell lysate was heated at 65°C for 30 min to kill vegetative bacteria prior to plating to determine colonies due to ungerminated spores.

To confirm that internalized spores represented a significant proportion of the cell-associated counts and also that intracellular germination was occurring, additional macrophages were preincubated with 5 μM cytochalasin D (Sigma) for 60 min prior to the addition of spores. Macrophages were then washed, lysed, and counted as described above.

Visualization of internalized spores by fluorescent confocal microscopy. In order to assess the ability of healthy macrophages to take up spores, spore internalization was qualitatively determined by confocal microscopy. *Bacillus anthracis* spores were labeled for detection by confocal fluorescence microscopy in the following manner. Bis-reactive, *n*-hydroxysuccinimido-ester Cy3 fluorophore (Amersham Life Sciences Inc.) was covalently conjugated to Sterne strain spores. Spores (1×10^9) were reacted with Cy3 dye in 0.1 M sodium carbonate buffer (pH 9.3) for 30 min at room temperature with occasional mixing according to the manufacturer's protocol for labeling proteins. Unconjugated dye was removed by centrifugation in ice-cold deionized water. Spore viability was not diminished by the fluorophore as assessed by plate counts. Human alveolar macrophages were cultured in the same media and density as described above for the spore and bacterial counts. However, for the purpose of microscopy, sterile glass coverslips were placed in the bottom of 12-well plates before the addition of cells. Cy3-labeled spores were added to the macrophages at an MOI of 5 for 60 min at 37°C. This MOI was chosen to enhance spore visualization and qualitative determination of internalization. Additional macrophages were pretreated with 5 μM cytochalasin D for 1 h prior to the addition of spores to provide another negative control for the visualization of spore internalization.

The cells were rinsed three times in PBS and fixed in freshly prepared 4% paraformaldehyde. The macrophages were permeabilized in 0.1% Triton X-100 in PBS for 5 min and then rinsed in PBS and stained for F-actin with 1 unit/coverslip Alexa Fluor 488-phalloidin (Molecular Probes). After two final rinses in PBS, the coverslips were mounted onto glass slides using ProLong Gold antifade reagent with DAPI (4′,6′-diamidino-2-phenylindole) (Molecular Probes). Fluorescent confocal laser scanning microscopy was conducted with a Zeiss LSM-510 META laser scanning confocal microscope using the OMRFQant imaging software.

RNA preparation and RPA. Human alveolar macrophages were plated into six-well culture plates at 1×10^6 cells/ml in RPMI medium with 2% FCS containing 50 μg/ml of gentamicin. This dose effectively prevented any detectable bacterial survival or replication as determined by plate counts, consistent with data reported previously by Pickering et al. (21). Thus, all the subsequent studies performed herein should represent the macrophage response to spores and not vegetative bacteria. Following overnight attachment, macrophages were stimulated with *Bacillus anthracis* spores (1 MOI) in the presence of gentamicin. Unstimulated control wells were prepared by exposing cells to equal volumes of spore diluent for 2 and 6 h. Cells were harvested by the addition of TRIzol reagent (Invitrogen), and the total RNA was isolated according to the manufacturer's protocol by using glycogen (20 mg/ml) as the carrier. Cells yielded 8 to 10 μg total RNA/well, 4 μg of which was used for a single RNase protection assay (RPA) reaction.

Relative gene expression was determined with the RiboQuant Multi-Probe RNase protection assay system (BD Biosciences/Pharmingen). Two template sets were used, the hCK5 set containing probes for lymphotactin, RANTES, inducible protein 10 (IP-10), macrophage inflammatory protein 1β (MIP-1β), MIP-1α, IL-8, and I-309/SCYA1 and a custom cytokine set containing probes for TNF-α, IL-12/p35, macrophage-derived chemokine (MDC) IL-10, gamma interferon (IFN-γ), IL-1β, granulocyte colony-stimulating factor, transforming growth factor β, and IL-6. Both template sets contained probes for ribosomal protein (L32) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for normalization for RNA loading. A labeled riboprobe was made with the In Vitro Transcription kit (BD Biosciences/Pharmingen) and [α-^{32}P]UTP. The RPA kit was used for hybridization of the probe with the target RNA in the samples and for digestion of unpaired transcripts. Additional controls included a sample containing baker's yeast tRNA, a sample with the hCK5 or custom template control RNA, and a sample with an unprotected probe. The resulting mRNA duplexes were separated on a standard 50-cm-long, 0.4-mm-thick polyacrylamide

gel. The gel was dried and imaged using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The image was analyzed with ImageQuant 5.0 software (Molecular Dynamics) using the volume quantitation method with histogram peak background subtraction. The identity of each protected band in a sample lane was determined from the position of the bands in the unprotected probe lane. The increase (*n*-fold) for each RNA species over control samples prepared at the nearest time points was determined after correction for loading using the L32 and GAPDH standards.

Infection of macrophages with spores for cytokine determination. After incubation of the macrophages overnight, the medium was removed, and fresh RPMI medium with 2% FCS and 50 μg/ml gentamicin was added to kill vegetative bacteria. Macrophages were exposed to Sterne spores at an MOI of 0.01 to 100 in triplicate wells of a 24-well plate and allowed to incubate at 37°C for 2.5 and 7.5 h. Gentamicin remained in the medium during the entire incubation time. Spore diluent was used as a negative control, and lipopolysaccharide (LPS) (1 μg/ml) was used as a positive control.

To determine the effect of inhibition of the signaling pathways ERK, p38, and JNK on cytokine induction, the specific inhibitors U0126, SB203580, and SP600125 (Calbiochem) were used (4, 5, 15). Macrophages were preincubated with the inhibitors at 10 and 25 μM in RPMI medium plus 0.2% FCS with gentamicin for 2 h at 37°C. The medium was replaced with RPMI–2% FCS with 50 μg/ml gentamicin, and the macrophages were exposed to *Bacillus anthracis* Sterne spores (MOI of 1) for 7.5 h at 37°C. The final concentration of the inhibitors was maintained throughout the experiment. LPS (1 μg/ml) was again used as a positive control, and mock-infected cells were treated with the inhibitor solvent dimethyl sulfoxide (DMSO). After incubation, the supernatants were collected, centrifuged at 10,000 × *g* for 2 min, removed to a new tube, and stored at −20°C.

For intracellular cytokine measurements, 0.5 ml of 0.05% deoxycholate was added to lyse the cells, the mixture was incubated at 37°C for 10 min, and the supernatant was stored at −20°C. Cytokine enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (2) by using anti-cytokine monoclonal primary capture antibodies (IL-1α clone 4414, IL-1β clone 8516, IL-6 clone 6708, and TNF-α clone 1825; R&D Systems) and biotinylated anti-cytokine polyclonal secondary detection antibodies (IL-1α, catalog no. AB-200-NA, IL-1β, catalog no. AB-201-NA, IL-6, catalog no. AB-206-NA, and TNF-α, catalog no. AB-210-NA; R&D Systems). Plates were developed using TMB reagent (BD Biosciences).

Signaling pathway kinase assay, SDS-PAGE, and immunoblotting. Human alveolar macrophages were harvested as described above, plated at a density of 1×10^6 cells/ml, and maintained overnight at 37°C in 5% CO_2 in 2 ml RPMI-1640 medium supplemented with 2% FCS and 50 μg/ml gentamicin. Prior to stimulation, the macrophages were washed twice with sterile PBS and preincubated at 37°C in 5% CO_2 for 4 h in serum-free growth medium containing gentamicin. Macrophage stimulation was conducted in 1 ml of serum-free medium plus gentamicin with Sterne spores (MOI of 1), phorbol 12-myristate 13-acetate (PMA) (100 ng/ml), or LPS (1 μg/ml). Mock-infected negative control cells were exposed to an equivalent volume of spore-free diluent. After incubation at 37°C in 5% CO_2 for the indicated times, the macrophages were harvested and lysed in 300 μl cold lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 10 mM each EDTA, NaF, Na-pyrophosphate, 1% NP-40, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 3 mM sodium vanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Macrophage homogenates were clarified by centrifugation at 4°C, and 20 to 30 μg of the resultant postnuclear lysates was mixed with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (60 mM Tris, pH 6.8, 10% glycerol, 2.3% SDS) and heated to 95°C for 5 min. The samples were separated by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. To detect activated, phosphorylated ERK1/2, p38, or SAPK/JNK, the membranes were blocked overnight in 5% powdered milk in Tris-buffered saline and then immunoblotted with specific affinity-purified rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA). Identically prepared membranes were probed with rabbit polyclonal anti-ERKl/2, anti-p38, or anti-SAPK/JNK antibodies that recognize both phosphorylated and nonphosphorylated forms of the signaling proteins (catalog no. 9101, 9102, 9211, 9212, 9251, and 9252; Cell Signaling Technology). The membranes were developed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cell Signaling Technology) and chemiluminescent reagents (Pierce Biotechnology, Rockford, IL). The developed membranes were exposed to X-ray film, and the digitally scanned film was quantified using Alpha Ease software (Alpha Innotech Corp., Leandro, CA). This included both the p42 and p44 bands for Erk quantification and the p46 and p54 bands for SAPK/JNK quantification.

Statistical analysis. Where applicable, the data were expressed as the means ± standard errors of the means (SEM). Statistical significance was determined by one-way analysis of variance (ANOVA) with Student-Newman-Keuls post hoc analysis. A P value of <0.05 was considered significant (31).

RESULTS

Assessment of *B. anthracis* spore internalization by cell-associated CFU. We first determined how rapidly primary human alveolar macrophages internalize *B. anthracis* spores. Human alveolar macrophages were derived from bronchoalveolar lavage fluid as described above (see Materials and Methods) and were exposed to spores with an MOI of 1. Spores were allowed to incubate for 15, 30, 60, and 120 min, after which the medium was removed and the cells were washed to remove spores not engulfed by the macrophages. The macrophages were then lysed, and CFU were determined by culture on LB agar. There was a steady increase in cell-associated CFU during the study, with a doubling time of 30 to 35 min (Fig. 1A). To determine whether this increase in the CFU was due to additional spore association with macrophages or to germination of bacteria, half of the cell lysates were heated to 65°C for 30 min. The resultant CFU represented only nongerminated spores. The decrease in CFU of heated lysates demonstrated that 85 to 90% of the spores had become vegetative bacteria by 15 min (Fig. 1B). A maximal macrophage cell-associated spore count was seen at 60 min and decreased by 120 min. This suggests that germination of spores associated with macrophages during this time was greater than that of additional spore association, although both likely contributed to the increase in CFU, as evidenced, at least at early times, by the initial increase in spore counts.

Cytochalasin D prevents actin cytoskeleton remodeling, and we used this reagent to confirm whether internalized spores were a significant component of the cell-associated counts or whether these counts represented primarily spores or bacteria adhering to the cell surface. The macrophages were preincubated with 5 μM cytochalasin D for 1 h before the addition of spores for 120 min. Spore counts were then made with heated and nonheated macrophage lysates as described above. The addition of cytochalasin D significantly decreased spore and bacterial counts by 80% (nonheated lysates). In addition, cytochalasin D appeared to decrease spore counts (heated lysates) by more than 60%, although this did not reach statistical significance. This suggests that most of the cell-associated counts represented internalized *B. anthracis* spores and internalized vegetative bacteria or germinated intracellular spores. The experiment does not exclude an indirect effect of cytochalasin D on cell-associated, macrophage-supported, extracellular germination.

These results suggest a rapid association of *Bacillus anthracis* spores and human alveolar macrophages with the cell internalization of spores. They also suggest a rapid germination of *B. anthracis* spores in human alveolar macrophages.

Visual confirmation of *B. anthracis* spore internalization by confocal microscopy. To qualitatively confirm that the increase in CFU seen in macrophage lysates represented internal *Bacillus anthracis* spores and bacteria and not just externally adherent spores, macrophages were incubated with Cy3-labeled Sterne ($pX01^+$ $pX02^-$) spores at an MOI of 5 CFU/macrophage for 60 min. The rinsed and fixed cells were stained with Alexa Fluor 488 phalloidin and DAPI and examined by con-

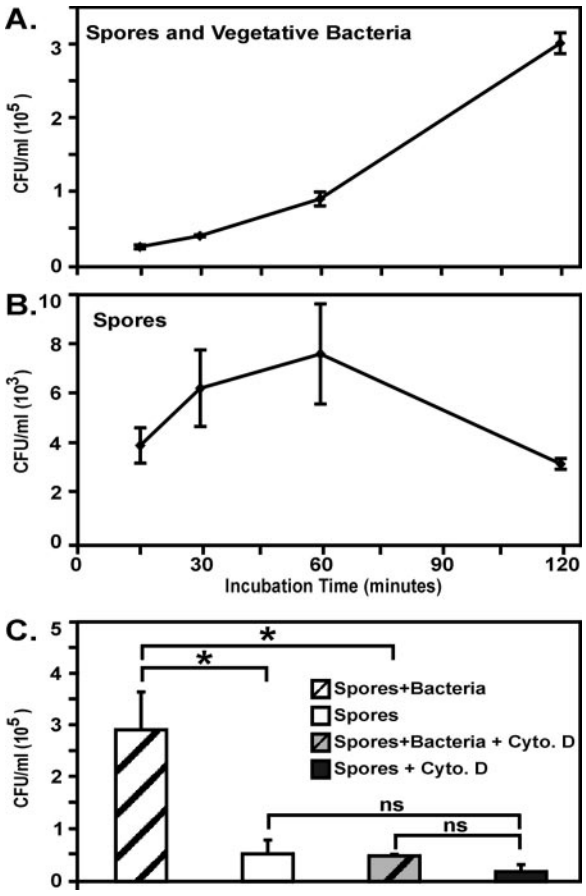

FIG. 1. Internalization of *Bacillus anthracis* spores by human alveolar macrophages. Human alveolar macrophages were exposed to Sterne strain 7702 for the times indicated at an MOI of 1 in the absence of gentamicin. The CFU inside the macrophages were determined by lysing with 0.05% deoxycholate and plating onto LB agar (see Materials and Methods). (A) CFU of the total number of spores and vegetative bacteria (unheated lysate). (B) Intracellular CFU of spores heated at 65°C to kill vegetative bacteria. (C) CFU of the total number of spores and vegetative bacteria (unheated lysate) or spore CFU (heated lysate) from untreated macrophages or macrophages pretreated with 5 μM cytochalasin D (Cyto. D) for 1 h prior to the addition of spores for 120 min. For all three panels, the data are expressed as the means ± SEM from three separate donor macrophage preparations. For panel C only, statistical significance was determined by ANOVA. The compared means are indicated by brackets. ns, not significant. $*P < 0.01$.

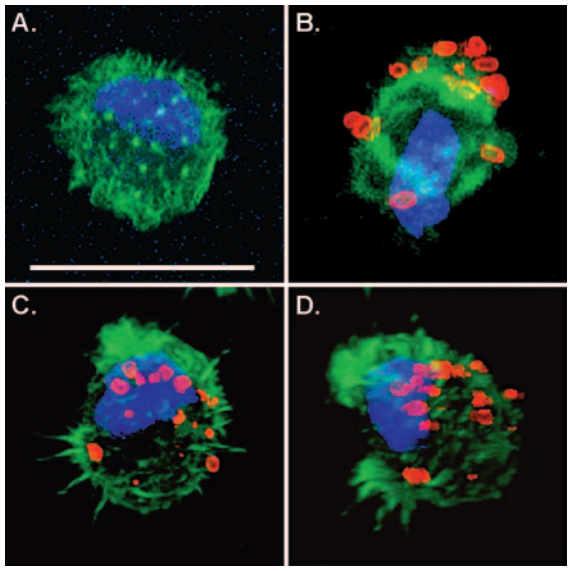

FIG. 2. Human alveolar macrophages phagocytose *Bacillus anthracis* Sterne spores. Human alveolar macrophages were exposed to Cy-3-labeled spores (red) at an MOI of 5 for 60 min in the absence of gentamicin, fixed, and stained for F-actin with Alexa Fluor 488 phalloidin (green) and for DNA with DAPI (blue). The results were imaged by confocal microscopy for red, blue, and green fluorescent emissions. (A) Macrophages exposed to spore diluent. (B) Macrophages pretreated with 5 μM cytochalasin D for 1 h prior to the addition of Cy3-labeled spores for 1 h. (C) Planar and (D) *x/y* rotational confocal images of a macrophage infected with Cy3-labeled Sterne spores are shown. Bar, 20 μm.

focal fluorescent microscopy (Fig. 2). Rotational images of stacked optical sections through the cells confirmed internalization of the spores (Fig. 2) (see the supplemental material). The number of spores taken up by individual macrophages varied, ranging from more than 10 spores internalized to as few as 1 or 2 spores internalized or none at all. Additional single (Fig. 2) and stacked (not shown) images of macrophages exposed to spores in the presence of cytochalasin D confirmed that the technique used is capable of distinguishing cell-associated spores from those that are internalized. Although these experiments were not designed to exactly determine the quantity of spores internalized, the results confirm qualitatively that human alveolar macrophages phagocytose *Bacillus anthracis* spores and that this is partially responsible for the increase in cell-associated CFU seen with continued incubation.

RNA expression of cytokines and chemokines by human alveolar macrophages exposed to *Bacillus anthracis* spores. We next examined the innate immune cytokine response of human alveolar macrophages to *Bacillus anthracis* spores using RPAs. Macrophages were exposed to spores (MOI of 1) or spore diluent as a negative control for 1 to 7 h. RPA for cytokines demonstrated a 10- to 35-fold induction of IL-6, IL-1β, TNF-α, transforming growth factor β, and granulocyte-macrophage colony-stimulating factor (GM-CSF) 3 h postinfection, with a peak at 5 h. IL-12/p35 levels increased within 1 h of infection with spores, which was followed by a very rapid increase of over 90-fold at 5 h. At times longer than 5 h, cytokine RNA induction declined but was still well over control values at 7 h postexposure (Fig. 3A).

RPA for chemokines also demonstrated RNA induction starting at 4 to 5 h postinfection, with levels either increasing throughout the time studied or decreasing slightly by 7 h postexposure. Monocyte chemotaxins were primarily induced, ranging from a 7- to 25-fold induction of MIP-1α, MIP-1β, I-309, and IP-10 to a 5-fold increase of monocyte chemoattractant protein 1 (MCP-1). In contrast, there was minimal induction of the lymphocyte chemotaxins RANTES and lymphotactin and the neutrophil chemotaxin IL-8 (Fig. 3B).

Thus, RPA results suggest a broad cytokine immune response but a specific monocyte chemokine response to exposure of human alveolar macrophages to *Bacillus anthracis* spores.

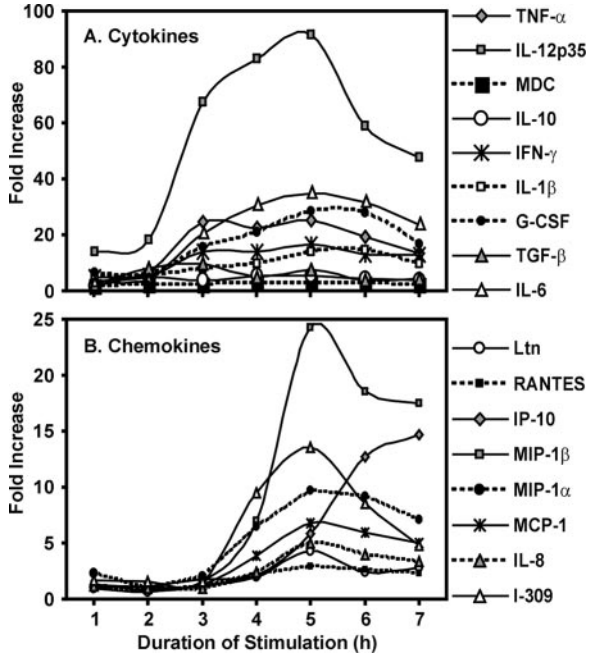

FIG. 3. *Bacillus anthracis* Sterne strain spores induce the production of mRNA for proinflammatory cytokines and chemokines by human alveolar macrophages. Human alveolar macrophages were exposed to spores (MOI of 1) in the presence of gentamicin for the times indicated. Equal volumes of spore diluent were used as a negative control. mRNA expression levels were measured by RPA using a custom cytokine template (A) and the hCK5 chemokine template (B) (Pharmingen) (see Materials and Methods). The increase in mRNA levels over controls (*n*-fold) was determined with normalization for levels of housekeeping genes in each sample. G-CSF, granulocyte colony-stimulating factor.

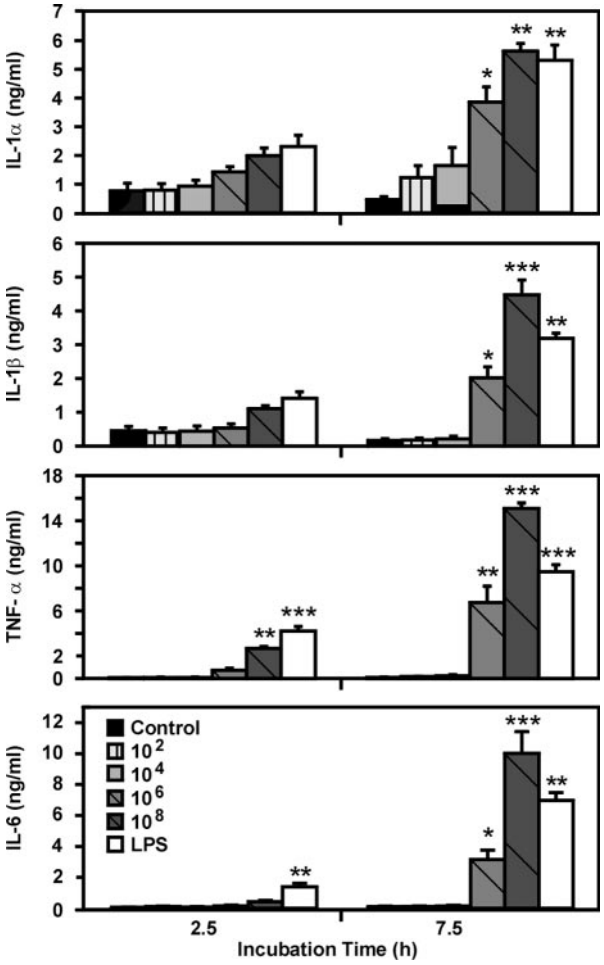

FIG. 4. *Bacillus anthracis* Sterne strain spores induce the production of proinflammatory cytokines by human alveolar macrophages. Human alveolar macrophages were exposed to increasing doses of spores in the presence of gentamicin at the times indicated. Spore buffer was used as a negative control, and LPS (1 μg/ml) was used as a positive control. Cytokine protein levels were determined on cell extracts (IL-1α and IL-1β) or supernatants (TNF-α and IL-6) by ELISA. Data are expressed as the means ± SEM from three separate donor macrophage preparations. Statistical significance was determined by ANOVA. Means were compared to data from the mock-infected control group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Induction of cytokines in human alveolar macrophages infected with *B. anthracis* spores. RPA data indicated an increase in the mRNA levels of several cytokines. To verify that this increase in endogenous RNA levels was reflected at the level of translation, we determined extracellular and intracellular protein levels at 2.5 and 7.5 h postinfection by ELISA. Macrophages were mock treated, treated with increasing doses of Sterne strain spores, or treated with LPS (1 μg/ml) as a positive control. Consistent with RPA results, we saw an increase in IL-1α, IL-1β, TNF-α, and IL-6 levels with spore exposure. Specifically, there was a dose-dependent increase in cytokine levels of intracellular IL-1α and IL-1β and secreted TNF-α or IL-6 at both 2.5 and 7.5 h postinfection. These levels were more evident at MOIs of ≥1 and at 7.5 h postinfection. At this time, the increases (*n*-fold) in IL-1α and IL-1β were 8- and 13-fold, respectively, at an MOI of 1 and 12- and 28-fold, respectively, at an MOI of 100. TNF-α and IL-6 levels at this time were increased 74- and 29-fold, respectively, at an MOI of 1 and 166- and 94-fold, respectively, at an MOI of 100. At an MOI of 100 after 7.5 h of exposure, these cytokines were stimulated to a greater extent than that seen with 1 μg/ml LPS (Fig. 4). We also tested for induction of IL-12 p35/70 using ELISA, but we saw only modest increases, perhaps due to the fact that both the RPA transcript and the ELISA protein levels (not shown) were at the lower limits of detection of the assays. There was significant baseline production of IL-8 protein without induction by *B. anthracis* spores as determined by ELISA, and this was consistent with IL-8 mRNA levels determined by RPA. There was no induction of cytokines when human alveolar macrophages (HAM) were exposed to undiluted supernatants of the final spore preparation, confirming that the active component of the spore preparation for cytokine induction was *B. anthracis* spores (data not shown).

These results show that the induction of cytokine genes, as determined by RPA, is consistently reflected at the protein level when the levels of these cytokines are easily detectable. The results also demonstrate a dose-dependent response to *Bacillus anthracis* spores and a threshold value at which macrophages respond to *Bacillus anthracis* spores.

Induction of MAPK signaling pathways in human alveolar macrophages exposed to *B. anthracis* spores. To determine the

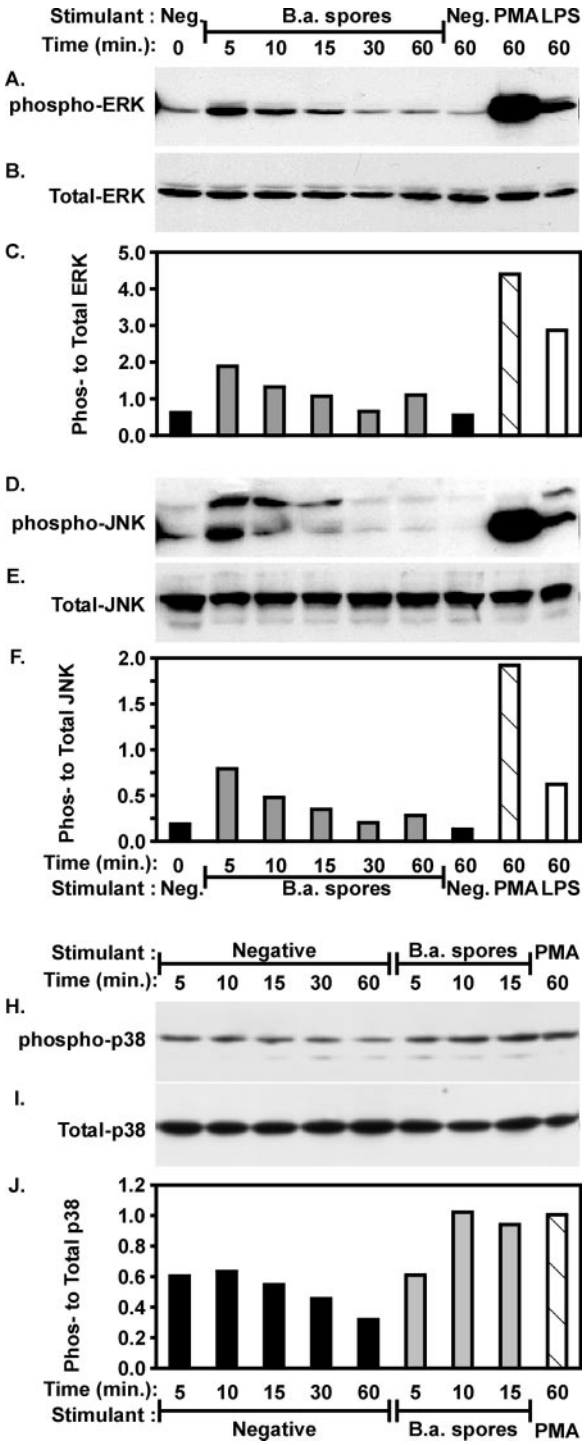

FIG. 5. Kinetics of ERK1/2, SAPK/JNK, and p38 phosphorylation by *Bacillus anthracis* Sterne strain spores (B.a.) in human alveolar macrophages. Serum-depleted human alveolar macrophages were exposed to Sterne spores at an MOI of 1 for the times indicated in the presence of gentamicin, macrophage lysates were prepared, and levels of total and phosphorylated ERK1/2 (A, B, and C), p38 (D, E, and F), and SAPK/JNK (H, I, and J) were assessed by Western analysis. PMA (100 ng/ml) and LPS (1 μg/ml) were used as positive controls, and spore diluent was used as a negative control. Western blots performed with antibody specific for phosphorylated ERK1/2 (A), p38 (D), or SAPK/JNK (H) are shown. Identically prepared blots were also probed with pan-anti-kinase antibody against ERK1/2 (B), p38 (D), or SAPK/JNK (H). Activation as determined by the ratio of phosphorylated kinase/total kinase for ERK1/2 (C), p38 (F), and SAPK/JNK (J) is graphed. The data shown are representative of two separate experiments.

role of signal pathway activation in cytokine induction by *B. anthracis* Sterne strain spores in human alveolar macrophages, we next studied the kinetics of spore-induced activation of the MAPK signaling cascades. We assessed ERK1/2, SAPK/JNK, and p38 activation as exhibited by phosphorylation in serum-starved human alveolar macrophages infected with Sterne spores at an MOI of 1. PMA (100 ng/ml) and LPS (1 μg/ml) stimulation for 60 min served as positive controls for phosphorylation, and mock-infected lysates were prepared at 0 and 60 min. Gentamicin (50 μg/ml) was present in the growth medium throughout the course of the experiment to prevent the growth of vegetative bacteria and the resultant production of anthrax toxins, which are known to cleave and inactivate MAPKs. At various times after infection, macrophage lysates were prepared, and total and phosphorylated ERK1/2, SAPK/JNK, and p38 levels were assessed by Western analysis. Phosphorylation was assessed by determining the ratio of phosphorylated kinase (Fig. 5A, D, and H) to total kinase (Fig. 5B, E, and I), and the resultant activities were graphed (Fig. 5C, F, and J). The ratio corrects for variations in the sample protein loaded onto SDS-PAGE gels. The results revealed that ERK1/2, SAPK/JNK, and p38 were activated by Sterne spores.

Phosphorylation of all three kinases peaked within 5 to 10 min of spore contact with the macrophages, with ERK1/2 and SAPK/JNK exhibiting 3.0- and 4.2-fold increases, respectively, over mock-infected controls at time zero. Spore-induced p38 phosphorylation at 10 min showed a more modest 1.5-fold increase over the negative control. The extent of phosphorylation seen in this negative control may have been caused by the additional manipulations required for these experiments, as physical stress activates p38 MAPK in macrophages (26). This is supported by the subsequent decrease in p38 MAPK phosphorylation in control cells during the time of incubation. By 30 min postinfection, the spore-induced activation of the all three kinases had reached their nadirs, declining to less than 1.5-fold above those of the negative controls. Spore-induced kinase activation increased slightly at 60 min postinfection, with ERK1/2, SAPK/JNK, and p38 phosphorylation reaching 2.0-, 2.2-, and 2.4-fold increases, respectively, over mock-infected negative controls at 60 min. These results indicate that exposure to *B. anthracis* Sterne spores elicits ERK1/2, SAPK/JNK, and p38 activation, which precedes cytokine mRNA and protein production in human alveolar macrophages.

Importance of multiple signaling pathways in cytokine induction by *Bacillus anthracis* spores. The findings described above demonstrate that the MAPK signaling pathways are activated when human alveolar macrophages are exposed to *B. anthracis* spores. We next sought to determine whether the activation of these signaling pathways is essential for the induction of cytokines. Macrophages were preincubated for 2 h in medium with 10 and 25 μmol of the ERK pathway inhibitor U0126, the p38 pathway inhibitor SB203580, or the SAPK/JNK pathway inhibitor SP600125. These doses were sufficient for

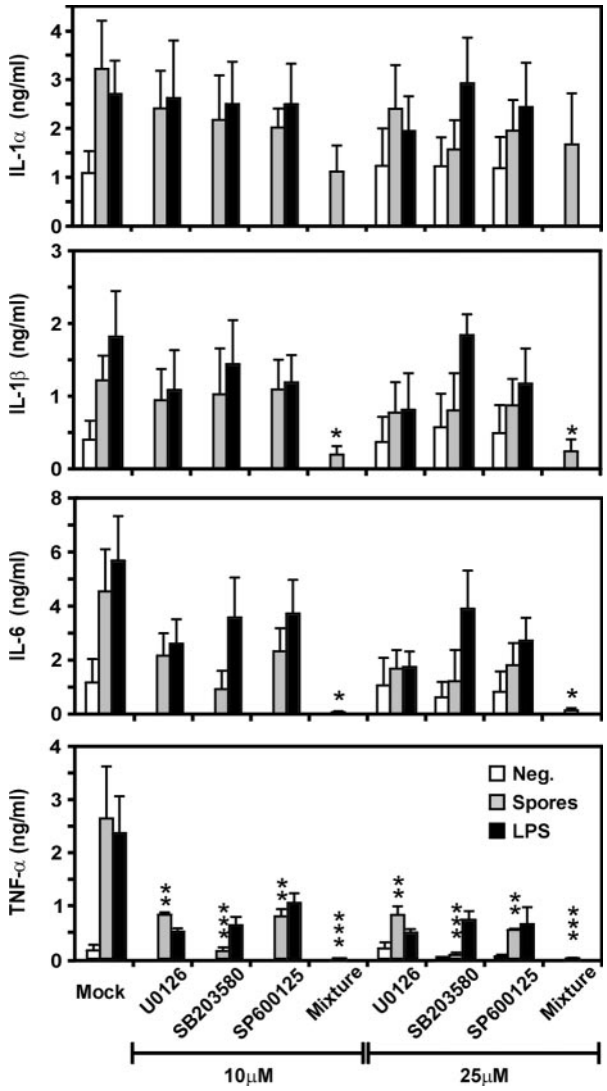

FIG. 6. Inhibition of *Bacillus anthracis* spore induction of cytokine proteins by signal pathway inhibitors. Serum-depleted human alveolar macrophages were preincubated with two doses, 10 and 25 μM, of the signaling pathway inhibitors U0126, SB203580, and SP600125, or all three inhibitors together (mixture), and exposed to spores (MOI of 1), spore-free inhibitor buffer (DMSO), or LPS (1 μg/ml) for 7.5 h in the presence of gentamicin prior to the measurement of the cytokines indicated by ELISA. The data are expressed as the means ± SEM of three experiments. Statistical significance was determined by ANOVA. Means were compared to data from the spore-infected control group without inhibitors. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

macrophages to inhibit induction of their corresponding signaling pathways by spores (not shown). A combination of the three inhibitors at both concentrations was also used. The inhibitors remained in the medium throughout the duration of the experiment, and cells that were not treated with inhibitors were exposed to an equivalent volume of inhibitor solvent (DMSO). The cells were incubated for 7.5 h either with spores at an MOI of 1 or with LPS prior to collection of the supernatants and cell lysates for cytokine determination.

The data reveal differences in the requirements for signaling pathway activation for the induction of specific cytokines by *Bacillus anthracis* spores (Fig. 6). Although individual pathway inhibitors appeared to diminish all four cytokine responses, only in the case of TNF-α was there statistically significant inhibition by a single inhibitor. The combination of multiple inhibitors was required to significantly inhibit IL-1β and IL-6 induction by spores, and, even in the presence of multiple inhibitors, the degree of inhibition of IL-1α, though suggestive, did not reach statistical significance. The effects of the signal pathway inhibitors on cytokine induction were not due to cytotoxicity, as cell viability determined by trypan blue exclusion was not altered by any dose or combination of inhibitors. *Bacillus anthracis* spore exposure alone also did not affect macrophage survival.

DISCUSSION

The alveolar macrophage is a focal point in the initial response of the innate immune system to pulmonary *B. anthracis* infection (21). As such, the response of macrophages to *B. anthracis* spores has been examined by several studies but has been limited to using differentiated peripheral blood monocytes or murine cell lines (1, 20, 21, 23). The current study examines the interaction of *Bacillus anthracis* spores with human alveolar macrophages obtained by bronchoalveolar lavage. We used the *B. anthracis* Sterne strain for these studies. We used bactericidal doses of gentamicin in the studies to focus on the response of macrophages to spores by eliminating vegetative bacteria (21). The Ames strain contains plasmids that encode both the production of toxins (pXO1) and the generation of capsule (pXO2), while the Sterne strain contains only the pXO1 plasmid. While the pXO2 plasmid contains genes mainly for the production of capsule, there are additional genes on the plasmid that are absent in the Sterne strain. This may affect some characteristics of the spore, and Welkos et al. previously reported that Sterne strain spores are "stickier" than Ames strain spores (29). There may also be some effects of pXO2 genes on anthrax pathogenesis, as there are virulence differences in pX01$^-$ strains in mice (30). On the other hand, our results using Sterne strain and HAM show effects of cytochalasin D similar to those seen with the Ames strain and mouse macrophages (29). Thus, the responses to the Sterne spores by HAM should be somewhat similar to those that occur with Ames strain. We also used the Sterne strain because it is far safer.

We performed additional studies in the absence of gentamicin to assess internalization and germination of *Bacillus anthracis*. In these studies, we show that spores associate with macrophages rapidly after contact with alveolar macrophages and, according to our studies using cytochalasin D, are rapidly internalized. Spore counts peaked at 60 min and were accompanied by increased CFU of heat-labile *Bacillus anthracis* spores. This suggests that there is significant germination of the spores by 60 min, which is similar to the germination time seen in murine macrophage-like RAW 264.7 cell lines (1, 6). The increase in total cell-associated CFU seen after 60 min suggests that either almost all of the spores rapidly germinate or there is significant replication of vegetative bacteria. If bacterial replication begins at a similar time after exposure to the cells as seen in RAW 264.7 cells and mouse primary peritoneal macrophages (3 h), then most of the increase in total cell-associ-

ated CFU should represent cell-associated germinated spores and not replicating vegetative bacteria (1, 21). Our study does not definitively answer this question, which would require additional investigation. As cytochalasin D significantly decreased cell-associated CFU at 120 min (Fig. 1), much of the germination or replication must be intracellular.

B. anthracis spores induce a robust proinflammatory cytokine response in human alveolar macrophages, as shown by our RPA and ELISA results. The mRNA levels of the cytokines IL-12p35, IL-6, GM-CSF, TNF-α, IFN-γ, and IL-1β increase at least 10-fold after 5 h of infection. The response of mouse primary peritoneal macrophages appears more limited, as only TNF-α and IL-6, but not IL-1β, IL-12p70, GM-CSF, or IFN-γ, are induced with spore exposure (21). The induction of cytokines by *Bacillus anthracis* spores has also been examined in peripheral blood monocytes differentiated to a human dendritic cell phenotype (3). In these cells, the cytokines TNF-α, IL-1α, IL-1β, and IL-6 as well as the chemokines IL-8 and RANTES, but not MCP-1, are induced. Our results using human alveolar macrophages show mainly induction of monocyte chemokines I-309, MIP-1, MCP-1, and IP-10 but minimal stimulation of the neutrophil chemotaxin IL-8 or the lymphocyte chemotaxins RANTES and lymphotactin. Some of the cytokine induction may not be a primary event of spore exposure but may be due to autocrine effects of cytokines directly induced by *B. anthracis* spores. The induction of monocyte chemotaxins is consistent with the monocytic infiltration seen in the lungs of patients with inhalational anthrax and likely plays a role in the recruitment of these cells (11). We do not expect that the response by alveolar macrophages represents the entire pulmonary innate immune cytokine response. There are, of course, other cells present in intact lungs that may independently provide an additional response or alter the response seen by alveolar macrophages. It is known, for example, that macrophage-epithelium interactions enhance the innate immune cytokine response to hyperoxia, particulants, and other toxins (8, 16, 17, 25, 28). We deliberately limited our studies to the interaction of spores with macrophages and diminished possible effects of germinated bacteria by using gentamicin in the medium. Gentamicin kills vegetative bacteria and therefore prevents the production of lethal toxin or edema toxin. It is possible that the absence of vegetative bacteria could have prevented other cytokines from being induced. However, toxins produced by the vegetative bacteria interfere with intracellular signaling pathways, and so the overall effect of the presence of these bacterial forms should be to diminish the cytokine response. MAPK pathways have been demonstrated to play a critical role in bacterial internalization and modulation of cytokine responses. In dendritic cells derived from peripheral blood monocytes, Sterne spores induced weak activation of ERK1/2 and strong activation of p38 but no activation of JNK after endocytosis (3). Our study is the first to report that all three MAPK families (ERK, p38, and JNK) are rapidly induced after *B. anthracis* infection (Fig. 5). This suggests that activation likely occurs during surface binding of spores to the alveolar macrophages. After the rapid induction, activation rapidly diminishes by 30 min of exposure. We do not think that this result is due to the action of bacterial LT because no CFU were detectable by plate assays in the presence of gentamicin. In addition, MEK2 cleavage did not occur in spore-exposed macrophages under these conditions (not shown). Our results using specific pathway inhibitors (U0126, SB203580, and SP600125) demonstrate that spore-induced MAPK activation is causally related to the induction of TNF-α, IL-6, IL-1β, and possibly IL-1α (Fig. 6). In the case of TNF-α, it appears that the activation of all three pathways is necessary for induction, as a single inhibitor prevents TNF-α induction. With IL-1β and IL-6, it appears that the activation of more than one pathway is required, because multiple pathways had to be blocked to inhibit the cytokine response. With regard to IL-1α, the lesser effect of the inhibitors, either alone or in combination, could be due to increased basal levels of IL-1α or additional non-MAPK pathways being involved in the induction of IL-1α.

Overall, our results demonstrate the activation of the innate immune cytokine system of primary human alveolar macrophages by *Bacillus anthracis* spores as evidenced by the induction of cytokines and chemokines. The chemokines induced are primarily monocyte, but not neutrophil or lymphocyte, chemotaxins. We also demonstrate a causal relationship between *Bacillus anthracis* spore-induced ERK, p38, and JNK activation and subsequent proinflammatory cytokine production. Our data also show that multiple signaling pathways are involved in cytokine induction. This is the first study to comprehensively examine the cytokine burst and signal pathway activation after *B. anthracis* Sterne strain infection of primary human alveolar macrophages.

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