Regulation by Jun N-terminal kinase/stress activated protein kinase of cytokine expression in *Mycobacterium avium* subsp *paratuberculosis*–infected bovine monocytes

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Objective—To evaluate activation of Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway in bovine monocytes after incubation with *Mycobacterium avium* subsp *paratuberculosis* (Mptb) organisms.

Sample Population—Bovine monocytes obtained from 4 healthy adult Holstein dairy cows.

Procedures—Bovine monocytes were incubated with Mptb organisms with or without a specific inhibitor of the JNK/SAPK pathway (SP600125) for 2, 6, 24, or 72 hours. Expression of interleukin (IL)-1β, IL-10, IL-12, IL-18; transforming growth factor-β (TGF-β); and tumor necrosis factor-α (TNF-α) and the capacity of Mptb-infected monocytes to acidify phagosomes and kill Mptb organisms were evaluated. Phosphorylation status of JNK/SAPK was evaluated at 10, 30, and 60 minutes after Mptb incubation.

Results—Compared with uninfected control monocytes, Mptb-infected monocytes had increased expression of IL-10 at 2 and 6 hours after incubation and had increased expression of TNF-α, IL-1β, IL-18, and TGF-β at 2, 4, and 6 hours. Additionally, Mptb-infected monocytes had increased expression of IL-12 at 6 and 24 hours. Addition of SP600125 (specific chemical inhibitor of JNK/SAPK) resulted in a decrease in TNF-α expression at 2, 6, and 24 hours, compared with untreated Mptb-infected cells. Addition of SP600125 resulted in a decrease in TGF-β expression at 24 hours and an increase in IL-18 expression at 6 hours. Addition of SP600125 failed to alter phagosome acidification but did enhance the capacity of monocytes to kill Mptb organisms.

Conclusions and Clinical Relevance—Activation of JNK/SAPK may be an important mechanism used by Mptb to regulate cytokine expression in bovine monocytes for survival and to alter inflammatory and immune responses. (*Am J Vet Res* 2006;67:1760–1765)

Abbreviations

Mptb	*Mycobacterium avium* subsp *paratuberculosis*
IL	Interleukin
TNF	Tumor necrosis factor
TGF	Transforming growth factor
MAPK	Mitogen-activated protein kinase
JNK/SAPK	Jun N-terminal kinase/stress-activated protein kinase
ERK	Extracellular signal regulated kinase
p38	p38 kinase
LPS	Lipopolysaccharide
RT-PCR	Reverse transcriptase-PCR
cDNA	Complementary DNA

Paratuberculosis is a chronic gastrointestinal infection of cattle and other ruminants caused by *Mycobacterium avium* subsp *paratuberculosis*.[1] The disease has a worldwide distribution and a high prevalence in domestic ruminants. This organism has also been implicated as the etiologic agent of segmental enteritis (Crohn's disease), a chronic inflammatory bowel disease of humans.[2]

Mycobacterium avium subsp *paratuberculosis* organisms are able to persist within macrophages in the small intestine despite the development of systemic, cellular, and humoral immune responses.[3] Results of previous studies[4-8] incriminate IL-10 as a possible mediator of immunosuppression associated with Mptb infection. Addition of a neutralizing anti–IL-10 antibody to bovine monocyte cultures before addition of Mptb organisms enables monocytes to kill greater than half of the organisms within 96 hours after incubation.[7] This is associated with increased phagosome acidification and increased nitric oxide production. Furthermore, addition of anti–IL-10 antibody increases expression of TNF-α and IL-12 mRNA. Neutralization of IL-10 also induces increased interferon-γ expression by peripheral mononuclear blood cells exposed to Mptb purified protein derivative.[9] Moreover, addition of exogenous IL-10 or TGF-β to bovine monocytes permits greater proliferation of Mptb organisms.[9]

Three subfamilies of MAPK, including JNK/SAPK, ERK-1 and -2, and p38, are described.[10-12] The MAPK pathway is activated by several extracellular stimuli including UV light, inflammatory cytokines, growth factors, and bacterial components.[13] The JNK/SAPK phosphorylates c-Jun, a component of the transcription factor AP-1.[13] In complex with other DNA binding proteins, activator protein-1 regulates the transcription of numerous genes including IL-2 and TNF-α. The JNK/SAPK also phosphorylates a variety of transcription factors such as activating transcription factor-2

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and Ets-like transcription factor-1[14,15] These transcription factors are involved in the regulation of several genes involved in immunity, notably proinflammatory genes (eg, TNF-α and IL-1β).

The purpose of the study reported here was to evaluate the activation of JNK/SAPK in bovine monocytes after Mptb incubation. A specific chemical inhibitor of JNK/SAPK was used to investigate the role of this pathway in Mptb-induced cytokine production and antimycobacterial functions of bovine monocytes.

Materials and Methods

Bacterial strain and culture conditions—*Mycobacterium avium* subsp *paratuberculosis* strain 505 was obtained from a cow with naturally acquired paratuberculosis evaluated at the Minnesota Animal Health Diagnostic Laboratory. Organisms were determined to be Mptb on the basis of dependence on mycobactin J for growth and detection of species-specific DNA sequences by use of a PCR assay.[16-18] Organisms were grown to a concentration of approximately 10^8 organisms/mL; washed; and resuspended in broth containing supplement,[a] Tween 80, mycobactin J, and 5% fetal bovine serum. Viability of organisms added to monocyte cultures varied between 78% and 93%, as determined by propidium iodide exclusion. Immediately before addition to monocyte cultures, organisms were washed and resuspended in medium.

JNK/SAPK specific inhibitor—A specific inhibitor of JNK/SAPK, SP600125,[b] was used in this study. This inhibitor blocks kinase activity but does not block phosphorylation of JNK/SAPK. In preliminary studies, concentrations from 3 to 10μM were evaluated and it was determined that optimal effects were observed with 10μM (data not shown). That concentration was used for the remainder of the study. Bovine monocytes viability, assessed by trypan blue exclusion, was not affected by this concentration of inhibitor (data not shown).

Cell isolation—Blood samples used for isolation of monocytes were collected from 4 healthy adult Holstein dairy cows that had negative test results for paratuberculosis, as determined by macrobacterial culture of fecal samples and PCR assay of fecal samples for paratuberculosis.[3,16] Peripheral blood mononuclear cells were isolated by centrifugation on a Percoll density gradient, as described.[6] Isolated cells were washed in Dulbecco PBS solution and resuspended at a concentration of 1×10^7 mononuclear cells/mL in RPMI medium containing 10% fetal bovine serum. For isolation of monocytes, 3×10^7 mononuclear cells were incubated in 60 × 15-mm tissue culture plates for 90 minutes at 37°C to allow cells to adhere. Nonadherent cells were removed by repeated washing with RPMI medium warmed to 37°C. Adherent cells were cultured overnight at 37°C in RPMI medium supplemented with 10% fetal bovine serum and 5% CO_2.

Culture of monocytes and organisms—*Mycobacterium avium* subsp *paratuberculosis* organisms (multiplicity of infection, 10 bacilli/monocyte) were added to cells, and incubation was continued at 37°C with 5% CO_2. Cellular mRNA was harvested from plates at 2, 6, or 24 hours by use of a commercial kit[c] following the manufacturer's instructions. Integrity of RNA preparations was assessed by use of RNA agarose gel electrophoresis, and RNA was frozen at –70°C until used.

Determination of JNK/SAPK phosphorylation by western blotting—A total of 1×10^6 bovine monocytes were incubated with 2×10^6 CFUs of Mptb. To eliminate endotoxin contamination, Mptb organisms were washed in LPS-free PBS solution. Additionally, some Mptb organisms were incubated with polymyxin B before addition to monocyte cultures (data not shown). As a positive control, monocytes were incubated with LPS (10 μg/mL) for 10, 30, or 60 minutes. For detection of phosphorylated JNK/SAPK, monocytes were washed with ice-cold Dulbecco PBS solution and cellular extracts were harvested with a lysis buffer[d] containing 50mM Tris-base, Dulbecco PBS solution, 137mM NaCl, 10% glycerol, 1% Nonidet P-40, 1mM NaF, leupeptin (5 mg/mL), aprotinin (5 mg/mL), and 2mM sodium orthovanadate. After addition of lysis buffer, the monocytes were incubated on ice for 5 minutes, scraped, and transferred to a 1.5-mL centrifuge tube. Lysates were centrifuged in a microcentrifuge at 14,000 × *g* for 10 minutes at room temperature (approx 25°C), and the supernatants were collected. Samples were loaded onto a 4% to 20% polyacrylamide gel and electrophoresed at 100 mA for 45 minutes. Proteins were transferred onto a polyvinyl diflouride membrane by wet blotting[e] for 2 hours. After blocking with 5% (wt/vol) nonfat dry milk in tris-buffered saline (0.9% NaCl) solution containing 0.1% Tween 20, membranes were incubated with primary antibodies for total JNK/SAPK[f] or phospho-JNK/SAPK[g] for 1 hour at room temperature. Blots were washed 3 times with Tween-Tris buffered saline solution (0.1% Tween 20 in 100mM Tris-HCL [pH, 7.5]; 0.9% NaCl) and incubated for 1 hour with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin. Membranes were developed by use of a chemiluminescence[h] and subsequently exposed to x-ray film for phosphorylation detection.

Determination of cytokines gene expression by RT-PCR assay—Genomic DNA was removed from mRNA samples. First-strand cDNA was synthesized by addition of random primers in 20 μL of reverse transcription mix (1× first-strand buffer, 10μM dithiothreitol, 500nM dinitrophenyl, 20 units of RNase inhibitor, and 100 units of RT). Then cDNA was diluted to 100 μL of total volume, and SYBR green master mix was added. Samples were analyzed in triplicate in a 96-well optical reaction plate. Each sample contained 5 μL of cDNA and 15 μL of SYBR green master mix. Primers were designed by use of a web-based program (**Appendix**).[i] Results were expressed as relative fold expression by use of the ΔC_T (ie, change in cycle to threshold) method. Glyceraldehyde-3-phosphate dehydrogenase was used to normalize results.

Ingestion and intracellular survival of mycobacterial organisms—Monocytes attached to coverslips were stained with Ziehl-Neelsen carbolfuchsin stain that stains mycobacteria and other acid-fast organisms. The percentage of monocytes containing organisms was determined by counting a minimum of 200 cells by use of light microscopy. Killing of organisms was assessed by use of a live-dead stain. This technique was chosen rather than the more standard serial dilution and colony counting assay because the particular organism used in this study grew poorly on solid media. After incubation with the organism for 72 hours, monocytes were washed twice in Dulbecco PBS solution and then monocytes were lysed by incubation with 0.1% deoxycholate for 5 minutes. The lysate was incubated with a 1:1 mixture of a green fluorescent stain and propidium iodine stain. Cells were placed on a microscope slide and examined on a fluorescent microscope (400× magnification) by use of a dual-band filter set that detects fluorescence in the green and red emission spectra. For this method, live organisms had green fluorescence and dead organisms had red fluorescence. At least 200 organisms were enumerated.

Acidification of phagosomes—Acidification of phagosomes was determined by use of a fluorescent technique. Organisms were labeled with fluorescein isothiocyanate before they were added to monocyte cultures. Bovine monocytes,

grown on 2 × 2-cm coverslips, were incubated with labeled mycobacteria (multiplicity of infection, 1:10) for 2 or 6 hours. A red stain[j] (final concentration, 50nM) was added during the last 30 minutes of incubation. The stain is taken up by acidified phagosome, where it is modified to become fluorescent. After incubation, coverslips were inverted onto glass slides and evaluated immediately by use of a confocal microscope. Intensity of green and red fluorescence was sequentially recorded at increments of 0.3µM throughout the depth of the cell. Sequential images were merged, intensity of green and red fluorescence for at least 200 phagosomes containing mycobacteria was quantified, and results were reported as a red-green colocalization coefficient. The colocalization coefficient was defined as the density of red fluorescence divided by the density of green fluorescence. Phagosomes that had approximately equal amounts of red and green fluorescence appeared yellow.

Statistical analysis—All tests were performed in triplicate, and results of at least 3 separate experiments were evaluated. Results were expressed as mean ± SD. Differences between cell cultures incubated with and without addition of JNK/SAPK inhibitor were analyzed by use of the paired Student *t* test. Values of $P < 0.05$ were considered significant.

Results

Activation of the JNK/SAPK pathway in monocytes infected in vitro with Mptb organisms was investigated. Bacterial LPS is known to activate JNK/SAPK and was used as a positive control. Monocytes were incubated with Mptb, and phosphorylation of JNK/SAPK was evaluated. Total JNK/SAPK was measured to control for proteins. Bovine monocytes incubated with Mptb had a > 3-fold increase in JNK/SAPK phosphorylation at all time points after infection, compared with untreated cells (**Figure 1**). Addition of polymyxin B to Mptb before addition to monocytes failed to alter JNK/SAPK phosphorylation (data not shown).

The JNK/SAPK regulation of cytokine expression by monocytes that ingest Mptb organisms was evaluated. Bovine monocytes were pretreated with the specific JNK/SAPK inhibitor, SP600125. This inhibitor blocks the phosphorylating capacity of JNK/SAPK but does not prevent phosphorylation of the JNK/SAPK by upstream kinases. Thereafter, cells were infected with Mptb, and selected proinflammatory and proimmune cytokines mRNA expressions were analyzed by RT-PCR assay.

The Mptb-infected monocytes upregulated IL-10 expression, compared with uninfected cells, at 2 and 6 hours after incubation (**Figure 2**). Treatment of Mptb-infected monocytes with SP600125 had no effect on IL-10 gene expression. Monocytes treated with Mptb upregulated TNF-α gene expression at 2, 6, and 24 hours. SP600125 downregulated the expression of TNF-α at all time points after treatment, compared with Mptb-infected cells (**Figure 3**). The Mptb-infected monocytes slightly upregulated the expression of IL-1β at 2, 6, and 24 hours after incubation (**Figure 4**). Treatment of Mptb-infected monocytes with SP600125 upregulated IL-1β > 50 fold at 2 hours and > 200 fold at 6 hours after incubation. The expression of IL-1β returned to basal levels at 24 hours after treatment in SP600125-treated cells. Cells treated with Mptb upregulated TGF-β at all time points after infection (**Figure**

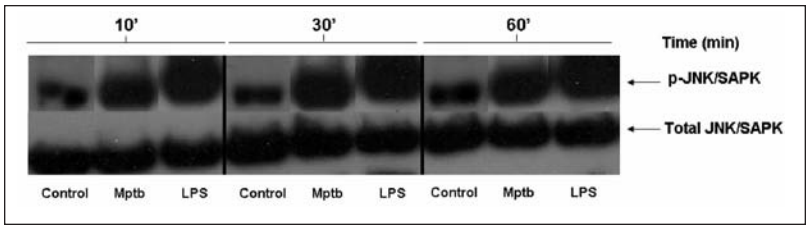

Figure 1—Activation of JNK/SAPK in bovine monocytes after treatment with Mptb. Bovine monocytes were incubated with Mptb at a ratio of 1:10 or with LPS (10 g/mL) for 10, 30, or 60 minutes and proteins were isolated for immunodetection. Similar data were obtained in 3 independent experiments.

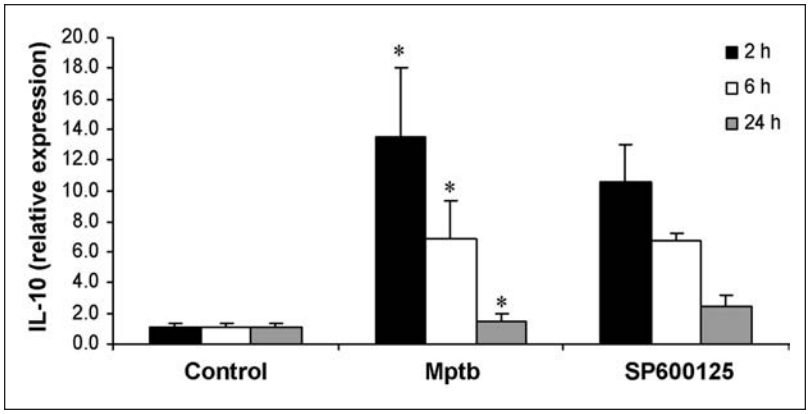

Figure 2—Effect of inhibition of the JNK/SAPK pathway on relative IL-10 mRNA expression by bovine monocytes at 2, 6, and 24 hours after Mptb incubation. Similar data were obtained in 3 independent experiments. *Significant ($P < 0.05$) difference between Mptb-infected monocytes and control monocytes.

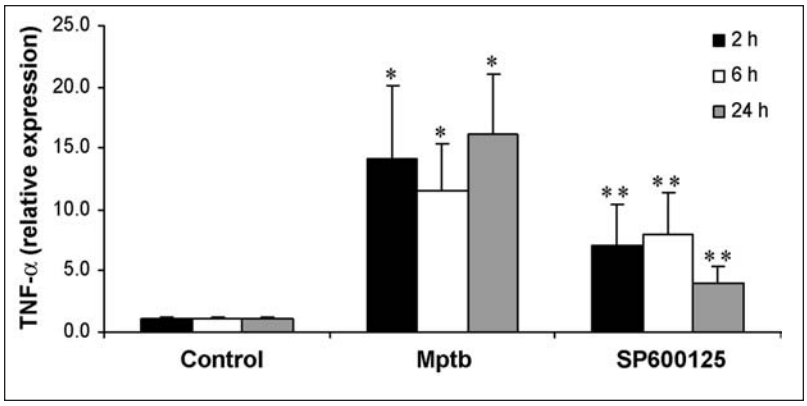

Figure 3—Effect of inhibition of the JNK/SAPK pathway on relative TNF-α mRNA expression by bovine monocytes at 2, 6, and 24 hours after Mptb incubation. **Significant ($P < 0.05$) difference between Mptb-infected monocytes treated with SP600125 and those that were not treated with SP600125. *See* Figure 2 for remainder of key.

5). Monocytes treated with SP600125 before addition of Mptb organisms had a marked decrease in the expression of TGF-β at 24 hours after infection. Bovine monocytes upregulated IL-18 at 2, 6, and 24 hours after addition of Mptb (**Figure 6**). Treatment with SP600125 resulted in increased IL-18 expression 6 hours after addition of Mptb organisms. The Mptb-infected monocytes upregulated IL-12 expression at 6 and 24 hours after incubation (**Figure 7**). Treatment with SP600125 upregulated IL-12 approximately 6-fold at 2 hours and 1.8 fold at 6 hours, compared with Mptb-infected monocytes; however, the expression of IL-12 significantly decreased at 24 hours.

To determine the effects of JNK/SAPK on the microbicidal activity of monocytes, phagosome acidification and the capacity of bovine monocytes to kill ingested organisms were analyzed. Treatment with SP600125 failed to alter organism phagocytosis (data not shown). Pretreatment with SP600125 resulted in no significant difference in acidification of phagosomes when cells were infected with Mptb (**Figure 8**). However, blocking this kinase pathway increased the capacity of macrophages to kill Mptb organisms at 72 hours after infection.

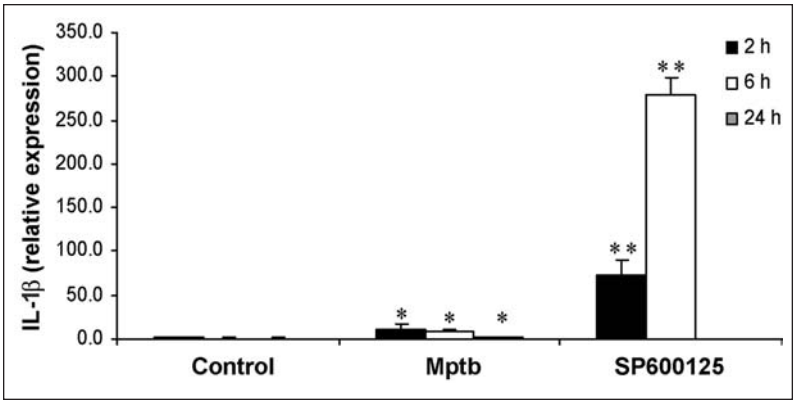

Figure 4—Effect of inhibition of the JNK/SAPK pathway on relative IL-1β mRNA expression by bovine monocytes at 2, 6, and 24 hours after Mptb incubation. *See* Figures 2 and 3 for remainder of key.

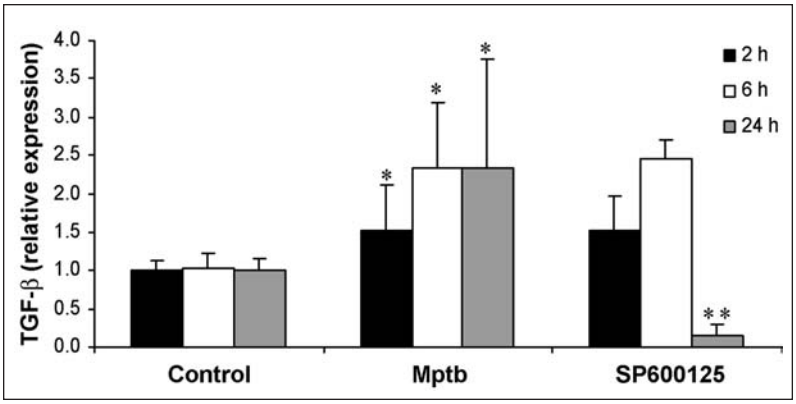

Figure 5—Effect of inhibition of the JNK/SAPK pathway on relative TGF-β mRNA expression by bovine monocytes at 2, 6, and 24 hours after Mptb incubation. *See* Figures 2 and 3 for remainder of key.

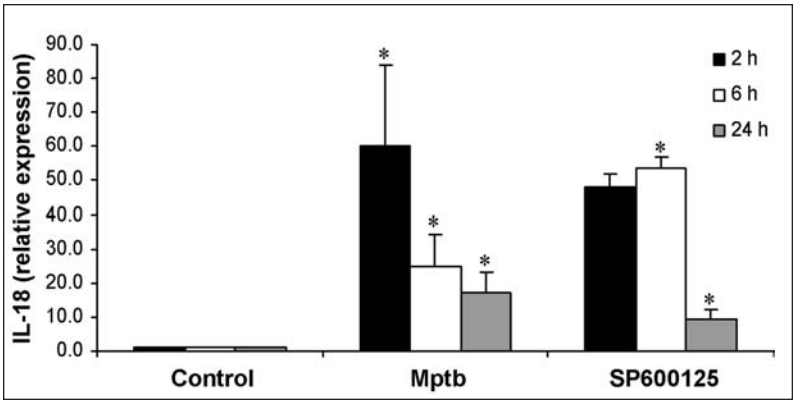

Figure 6—Effect of inhibition of the JNK/SAPK pathway on relative IL-18 mRNA expression by bovine monocytes at 2, 6, and 24 hours after Mptb incubation. *See* Figure 2 for remainder of key.

Discussion

Results of our study indicate that Mptb alters immune cytokine expression and bovine monocyte bactericidal functions by activating the JNK/SAPK pathway. Pathogenic organisms, such as *Mycobacteria* spp, *Leishmania* spp, and *Toxoplasma* spp, have developed mechanisms to circumvent macrophage antimicrobial activities.[19-21] Uncovering the mechanisms by which these organisms disrupt macrophage responses and attenuate effective innate and adaptive immune responses is critical in designing novel therapies and vaccines.

Phosphorylation of the MAPK pathway leads to activation of a variety of transcription factors that are important in the initiation of antimicrobial responses and production of immune cytokines.[22] Mycobacterial infection has been shown to be associated with phosphorylation of MAPK kinases.[23] In a study[23] on human monocytes, ERK-1 and -2 and p38 pathways were needed for *Mycobacterium tuberculosis*–induced TNF-α production, whereas only the p38 pathway was needed for IL-10 production. Previous results in our laboratory have demonstrated that p38 is phosphorylated at early time points after Mptb ingestion by bovine monocytes, suggesting a role of this kinase in cell-signaling events associated with organism invasion.[24] Compared with ERK-1 and -2 and p38, relatively little is known about the role of the JNK/SAPK pathway in mycobacterial infections.

In our study reported here, blocking the JNK/SAPK pathway significantly changed the expression of key proinflammatory cytokines. Interleukin-1β, IL-18, and IL-12 expression increased at early time points after infection, when the JNK/SAPK pathway was blocked. These data indicate that Mptb-associated activation of JNK/SAPK may suppress important cell-signaling pathways or transcrip-

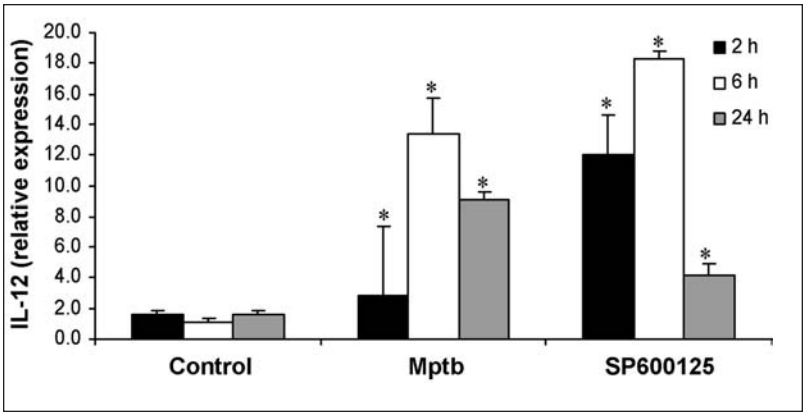

Figure 7—Effect of inhibition of the JNK/SAPK pathway on relative IL-12 mRNA expression by bovine monocytes at 2, 6, and 24 hours after Mptb incubation. *See* Figure 2 for remainder of key.

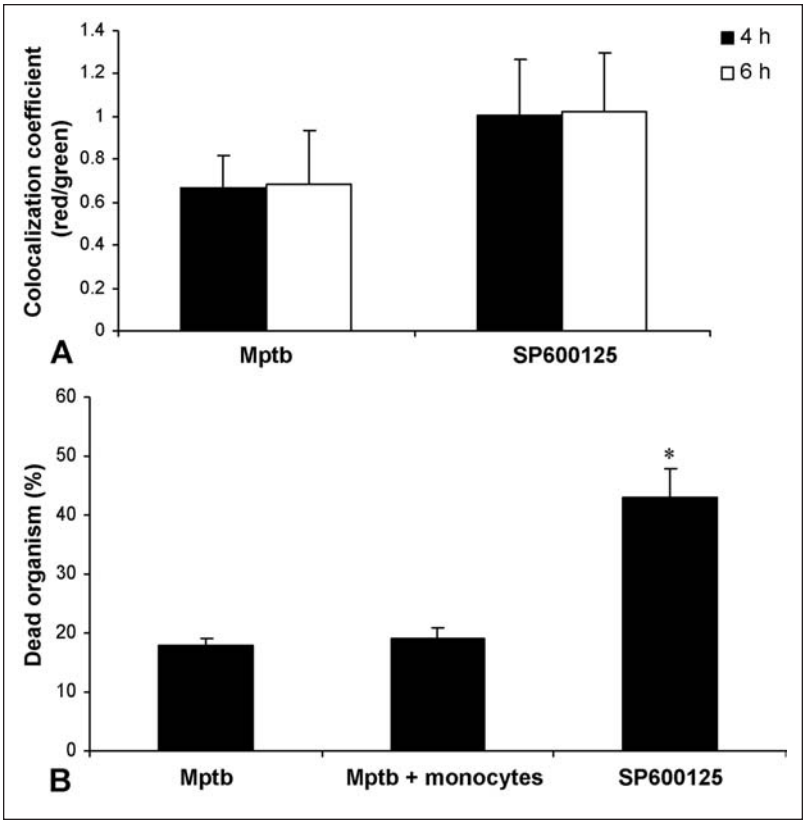

Figure 8—Role of JNK/SAPK in the capacity of Mptb-infected monocytes to acidify phagosomes (A) and kill Mptb organisms (B). Similar data were obtained in 3 independent experiments. *Significantly ($P < 0.05$) different from control values.

tion factors involved in proinflammatory and proimmune responses. This might be an important mechanism used by Mptb to manage to survive and proliferate shortly after the infection.

An interesting finding in our study was that SP600125 did not affect IL-10 expression. It has been shown that p38 is a key kinase involved in the overexpression of IL-10 in mycobacterial infection.[25,26] The JNK/SAPK is structurally similar to p38 and was expected to have overlapping roles with this kinase on IL-10 expression. On the other hand, Mptb upregulated TGF-β, a cytokine implicated in immune modulation toward a T-helper 2 cell-type immune response. Inhibition of JNK/SAPK activation downregulated TGF-β, suggesting that this cell signaling might be important for the ability of Mptb to infect and survive on monocytes.

Interleukin-12 is a major proimmune cytokine produced by mononuclear phagocytes in response to bacteria and bacterial products.[27-30] Unlike results in our previous study,[8] Mptb-infected monocytes upregulated IL-12 mRNA. This difference may be attributable to the use of a different Mptb strain in the study reported here. Inhibiting JNK/SAPK further upregulated IL-12 mRNA expression in Mptb-infected monocytes at 2 and 6 hours after incubation. This effect could be the result of a direct effect of JNK/SAPK on IL-12 transcription or to an indirect effect mediated by other cytokines or signaling pathways. The inhibition of IL-12 expression might be a mechanism used by Mptb to regulate the T-helper 1 cell (stimulated by IL-12) toward a T-helper 2 cell-type immune response. This might have implications on the overall ability of the organisms to survive on monocytes.

The MAPK family has been incriminated in the arrest of *M tuberculosis* phagosome maturation.[31] The MAPK has been associated with reduced recruitment of endosomes and phagosomal membrane–tethering molecules responsible for a proper phagosome-lysosome fusion. Our results indicate that blocking the JNK/SAPK pathway fails to significantly alter phagosome acidification but does increase the capacity of macrophages to kill Mptb organisms. Further studies are needed to understand the complex molecular events involved in these mechanisms.

Overall, we studied the role of JNK/SAPK in the induction of cytokine expression and key antimycobacterial mechanisms in bovine monocytes ingesting Mptb organisms. Addition of Mptb to monocytes resulted in a sustained increase of JNK/SAPK phosphorylation at early time points after in vitro infection. Inhibition of JNK/SAPK resulted in modulation of key proimmune and proinflammatory cytokines but minimal change in anti-inflammatory cytokines. Increased organism killing was observed when the JNK/SAPK pathway was blocked, indicating that the JNK/SAPK pathway may attenuate antimicrobial activity.

Taken together, these data indicate that signaling through JNK/SAPK may be a pathway used by Mptb to attenuate the immune response by selectively modulating release of proinflammatory and proimmune

cytokines. These findings indicate that the molecular manipulation of JNK/SAPK may be useful in designing new therapies or adjuvants for development of effective Mptb vaccines.

a. OADC, Difco Labs, Detroit, Mo.
b. SP600125, Calbiochem, La Jolla, Calif.
c. RNeasy kit, Qiagen, Valencia, Calif.
d. Cell lysis buffer, Cell Signaling, Beverly, Mass.
e. ECL blotting buffer, Pierce, Rockford, Ill.
f. Clone 9252, Cell Signaling, Beverly, Mass.
g. Clone 9345, Cell Signaling, Beverly, Mass.
h. BackLight kit, Invitrogen, Carlsbad, Calif.
i. Whitehead Institute for Biomedical Research. Available at: frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Accessed November 15, 2005.
j. LysoTracker Red, Invitrogen, Carlsbad, Calif.

Appendix

Primers used for RT-PCR assay.

Gene	Primer
TNF-α	Forward 5′–TCAAACACTCAGGTCCTCTTCTCA–3′
	Reverse 5′–GTCGGCTACAACGTGGGCTACC–3′
IL-10	Forward 5′–CGGCTGCGGCGCTGTCATC–3′
	Reverse 5′–TCACCTTCTCCACCGCCTTGCTCT–3′
GAPDH	Forward 5′–GAAACCTGCCAAGTATTGATGAGAT–3′
	Reverse 5′–TGTAGCCTAGAATGCCCTTGAGAG–3′
IL-18	Forward 5′–TTTGGCAAACTTGAACCTAAGC–3′
	Reverse 5′–ACAGTCAGAATCAGGCATATCC–3′
IL-1β	Forward 5′–TTGGAGAAGGAAATGGCAAC–3′
	Reverse 5′–TTCAGTCGTGTCCGACTCTG–3′
TGF-β	Forward 5′–CTGAGCCAGAGGCGGACTAC–3′
	Reverse 5′–TGCCGTATTCCACCATTAGCA–3′
IL-12 (p40)	Forward 5′–TCGGCAGGTGGAGGTCA–3′
	Reverse 5′–ACACAAAACGTCAGGGAGAAGTAG–3′

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

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