Role of the MAPK^{ERK} pathway in regulation of cytokine expression by *Mycobacterium avium* subsp *paratuberculosis*–exposed bovine monocytes

Cleverson D. Souza, DVM, MS; Oral A. Evanson, BS; Douglas J. Weiss, DVM, PhD

Objective—To evaluate the role of the mitogen-activated protein kinase extracellular signal-regulated kinase (MAPK^{ERK}) pathway in the interaction between *Mycobacterium avium* subsp *paratuberculosis* (MAP) organisms and bovine monocytes.

Sample Population—Monocytes obtained from healthy adult Holstein dairy cows that were not infected with MAP organisms.

Procedures—Monocytes and MAP organisms were incubated together with or without a specific inhibitor of the MAPK^{ERK} pathway (PD98059), and the capacity of monocytes to express tumor necrosis factor alpha (TNF)-α and interleukin (IL)-10 and -12, produce nitric oxide, acidify phagosomes, kill MAP organisms, and undergo apoptosis was evaluated.

Results—The MAPK^{ERK} pathway was activated within 10 minutes after addition of MAP organisms to monocytes. Addition of PD98059 to monocyte-MAP mixtures decreased monocyte TNF-α and IL-12 mRNA expression but had no effect on IL-10 mRNA expression. Treatment with PD98059 failed to induce significant alterations in phagosome acidification, organism killing, nitric oxide production, or apoptosis of MAP-exposed monocytes.

Conclusions and Clinical Relevance—Results indicated that the MAPK^{ERK} pathway was activated during the interaction of MAP organisms with monocytes, which initiated TNF-α and IL-12 mRNA expression but failed to initiate antimicrobial activity. The MAPK^{ERK} pathway may be involved in initiating proinflammatory and proimmune responses in MAP infection in cattle. (*Am J Vet Res* 2007;68:625–630)

Mycobacterium avium subsp *paratuberculosis* is the etiologic agent of paratuberculosis (also known as Johne's disease), a severe chronic enteritis of ruminants.[1] On entering the oral cavity, MAP organisms target mucosa-associated lymphoid tissues, where they are phagocytosed and multiply within macrophages and dendritic cells.[2-5] The mechanisms by which MAP organisms successfully colonize macrophages are incompletely understood.

Several cell signaling pathways have been incriminated in the interaction of macrophages with *Mycobacterium* organisms.[6] Two of the most studied pathways involve MAPK and nuclear factor-κB.[6-8] The MAPKs are a family of serine-threonine protein kinases that are composed of 3 major enzymes: MAPK^{ERK}, MAPK^{p38}, and MAPK^{JNK}.[9] In previous in vitro studies,[10,11] MAPK^{p38} and MAPK^{JNK} were activated at early time points after infection of bovine monocytes with MAP organisms. The MAPK^{p38} induced expression of the anti-inflammatory cytokine IL-10 and inhibited organism killing and phagosome acidification. Alternatively, MAPK^{JNK} induced expression of TNF-α and prevented killing of MAP organisms.[11] Overall, results of these studies indicate that the MAPK^{p38} and MAPK^{JNK} signaling pathways may contribute to survival and proliferation of MAP organisms within mononuclear phagocytes.

Abbreviations

MAP	*Mycobacterium avium* subsp *paratuberculosis*
MAPK	Mitogen-activated protein kinase
MAPK^{ERK}	Mitogen-activated protein kinase extracellular signal-regulated kinase
MAPK^{p38}	Mitogen-activated protein kinase-p38
MAPK^{JNK}	Mitogen-activated protein kinase c-jun N-terminal kinase
IL	Interleukin
TNF-α	Tumor necrosis factor-α

The purpose of the study reported here was to evaluate the role of the MAPK^{ERK} pathway in the interaction between MAP organisms and bovine monocytes. To this end, a specific chemical inhibitor of MAPK^{ERK} was used to investigate the role of this pathway in the MAP-induced cytokine production and anti-mycobacterial responses of bovine monocytes.

Materials and Methods

Bacterial strain and culture conditions—*Mycobacterium avium* subsp *paratuberculosis* (strain 166) was obtained from a cow with naturally acquired paratuberculosis that was evaluated at the Minnesota Ani-

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From the Department of Veterinary and Biomedical Science, University of Minnesota, Saint Paul, MN 55108.
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Address correspondence to Dr. Weiss.

mal Health Diagnostic Laboratory. The organisms were determined to be MAP on the basis of their dependency on mycobactin J for growth and on detection of species-specific DNA sequences by use of a standard PCR assay.[12] The organisms were grown to a concentration of approximately 10^8 mycobacteria/mL, washed, and resuspended in a combination of broth medium,[a] Tween 80,[b] mycobactin J,[c] and 5% fetal bovine serum.[c] Viability of the organisms varied between 78% and 93%, as determined by propidium iodide[d] exclusion. Immediately before addition to monocyte cultures, organisms were washed and resuspended in culture medium.

$MAPK^{ERK}$ pathway specific inhibitor—A specific inhibitor of the $MAPK^{ERK}$ pathway,[13] PD98059,[d] was used in the study. This inhibitor blocks phosphorylation of MEK1, which is the kinase that specifically phosphorylates $MAPK^{ERK}$. The specificity of PD98059 for MEK1 has been extensively evaluated.[13] When used at concentrations of 1 to 20µM, PD98059 is highly specific for MEK1 in a variety of experimental models both in vitro and in vivo.[13] In preliminary studies, we evaluated concentrations of PD98059 ranging from 1 to 10µM and determined that optimal effects were achieved at a concentration of 3µM (data not shown). This concentration was used for the remainder of the study. Viability of monocytes in culture, as determined by trypan blue exclusion, was not affected by addition of PD98059 alone (data not shown). Additionally, phagocytosis of MAP organisms, as determined by use of light microscopy after staining with acid-fast staining, was not affected by addition of PD98059.

Cell isolation—Blood samples (300 mL each) were collected from 5 healthy adult Holstein dairy cows that belonged to the University of Minnesota dairy herd. The animals in this herd were regularly tested for paratuberculosis and were known to be negative for MAP infection on the basis of results of microbial culture of feces and serum ELISA. Peripheral blood mononuclear cells were isolated by use of a density gradient centrifugation medium,[b] as described.[14] 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 monocytes, and incubation was continued at 37°C and 5% CO_2. The mRNA was harvested from plates at 2, 6, or 24 hours by use of a commercial kit.[e] Integrity of each RNA preparation was assessed by use of RNA agarose gel electrophoresis.

Determination of $MAPK^{ERK}$ phosphorylation via western blotting—After incubation with MAP organisms for 10, 30, or 60 minutes, monocytes were washed with ice-cold Dulbecco PBS solution and cellular extracts were harvested by use of a lysis buffer containing 50mM Tris-base, Dulbecco PBS solution, 137mM NaCl, 10% glycerol, 1% nonylphenylpolyethylene glycol, 1mM sodium fluoride, leupeptin (5 mg/mL), aprotinin (5 mg/mL), and 2mM sodium orthovanadate.[f] After addition of the lysis buffer, monocytes were incubated on ice for 5 minutes, scraped, and transferred to a 1.5-mL centrifuge tube. Lysates were centrifuged at 14,000 × *g* for 10 minutes at room temperature (25°C), and supernatants were collected. Samples were loaded onto a 4% to 20% polyacrylamide gel and underwent electrophoresis at 15 mA for 45 minutes. Proteins were transferred to a polyvinyldifluoride membrane[g] by wet blotting at 15 mA for 1.5 hours. After blocking with 5% (wt/vol) nonfat dry milk[b] in Tris-buffered saline solution containing 0.1% Tween 20, membranes were incubated overnight at 4°C with anti-mouse $MAPK^{ERK}$[h] or anti-mouse phosphorylated $MAPK^{ERK}$.[i] The amino acid sequences of mouse and bovine $MAPK^{ERK}$ were compared. The alignment indicated 80% homology between the mouse and bovine sequences. Moreover, the phosphorylated antibody-binding site on $MAPK^{ERK}$ was identical between mouse and bovine sequences. Blots were washed 3 times with Tween-Tris buffered saline solution (0.1% Tween 20 in 100mM Tris-HCL with 0.9% NaCl; pH, 7.5) and were incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin.[g] Membranes were developed by use of a chemiluminescence assay and subsequently exposed to radiographic film for detection of phosphorylation.[g] To eliminate possible endotoxin contamination, MAP organisms were resuspended in endotoxin-free PBS solution. Additionally, some MAP organisms were incubated with polymyxin B[b] before addition to monocyte cultures (data not shown).

Determination of cytokine gene expression via real-time PCR assay—Genomic DNA was removed from mRNA samples by use of a commercial kit[j] following the manufacturer's instructions. 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 reverse transcriptase). Then, cDNA was diluted to 100 µL total volume and SYBR green master mix was added. Samples were analyzed in triplicate in a 96-well optical reaction plate.[k] Each sample contained 5 µL of cDNA and 15 µL of SYBR green master mix.[k] Primers were previously synthesized (**Appendix**).[10] Results were expressed as relative fold expression by use of the Delta cycle threshold method.[15] Glyceraldehyde-3-phosphate dehydrogenase expression was used to adjust (normalize) the results. Preliminary results indicated no variation in the expression of glyceraldehyde-3-phosphate dehydrogenase in MAP-exposed monocytes treated with PD98059, compared with untreated MAP-exposed monocytes.

Phagocytosis and intracellular survival of MAP organisms—Monocytes were incubated with MAP organisms for 2 hours before staining with Ziehl-Neelsen carbolfuchsin stain.[b] The percentage of monocytes containing organisms was determined by counting a mini-

mum of 200 cells by use of a light microscope. Killing of organisms was assessed by use of a live-dead stain.[l] This technique was chosen rather than use of the more standard serial dilution and colony-counting assay because the particular organism used in the study grew poorly on solid media. After incubation with the organism for 72 hours, monocytes were washed twice in Dulbecco PBS solution and 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.[d] Cells were placed on a microscope slide and examined by use of a fluorescent microscope[m] at 400× magnification with a dual-band filter set that detected 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 counted.

Acidification of phagosomes—*Mycobacterium avium* subsp *paratuberculosis* organisms were labeled with fluorescein isothiocyanate before being added to monocyte cultures. Bovine monocytes (grown on 2 × 2-cm coverslips) were incubated with fluorescein-labeled mycobacteria (multiplicity of infection, 10 bacilli/monocyte) for 6 hours. An acidotropic probe,[l] at a final concentration of 50nM, was added during the last 30 minutes of incubation. This stain is modified to become fluorescent within acidified phagosomes in live cells. After incubation, the coverslips were inverted onto glass slides and evaluated immediately by use of a confocal microscope.[m] Intensity of green and red fluorescence was sequentially recorded at increments of 0.3 µm throughout the depth of the cell. Sequential images were merged, and intensity of green and red fluorescence for at least 200 phagosomes containing mycobacteria was quantified; 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.

Nitric oxide production—After 6 and 24 hours of incubation, nitrite (ie, the stable by-product of nitric oxide) concentration was measured in culture supernatants. Culture supernatants were mixed with 200 µL of Griess reagent (1% sulfanilamide, 0.1% napthylethylenedamine dihydrochloride, and 2.5% H_3PO_4) and incubated at 25°C for 10 minutes. Absorbance was determined at 540 nm, and absorbance readings were converted to micromolar concentrations by comparison of results with a standard curve generated with $NaNO_2$.

Apoptosis—Apoptosis of bovine monocytes was evaluated by use of a nuclear staining technique.[16,n] Other methods to detect apoptosis were not used because of technical problems with the assays. Staining of bovine monocytes with Annexin V yields negative results, and the terminal deoxynucleotidyltransferase-mediated uridine triphosphate-biotin nick end-labeling assay reportedly yields inconsistent results.[16] Monocytes were incubated with and without MAP organisms (multiplicity of infection, 10 bacilli/monocyte) for 24 hours. For some cultures, PD98059 was added 1 hour before incubation with MAP organisms. Culture plates were then incubated with a fluorescent stain[n] for 10 minutes.[16] Cultures were examined by use of a fluorescence microscope[m] with excitation at 350 mm and emission at 461 mm. A minimum of 200 cells was counted on each 2 slides, and the mean percentage of fluorescent nuclei was determined.

Statistical analysis—All tests were done in triplicate, and results of at least 3 separate experiments were evaluated. Results are expressed as mean value ± SD. Differences between cell cultures incubated with and without addition of PD98059 were analyzed by use of the paired student *t* test. Values of $P < 0.05$ were considered significant.

Results

MAP-induced signaling through the MAPK^{ERK} pathway—Monocytes were incubated with MAP organisms for 10, 30, or 60 minutes and phosphorylated (**Figure 1**); total MAPK^{ERK} expression was then determined (measured as arbitrary units). *Mycobacterium avium* subsp *paratuberculosis* induced a 2.5-fold increase in the MAPK^{ERK} phosphorylation at 10 minutes after addition to monocytes, compared with control values (2.5

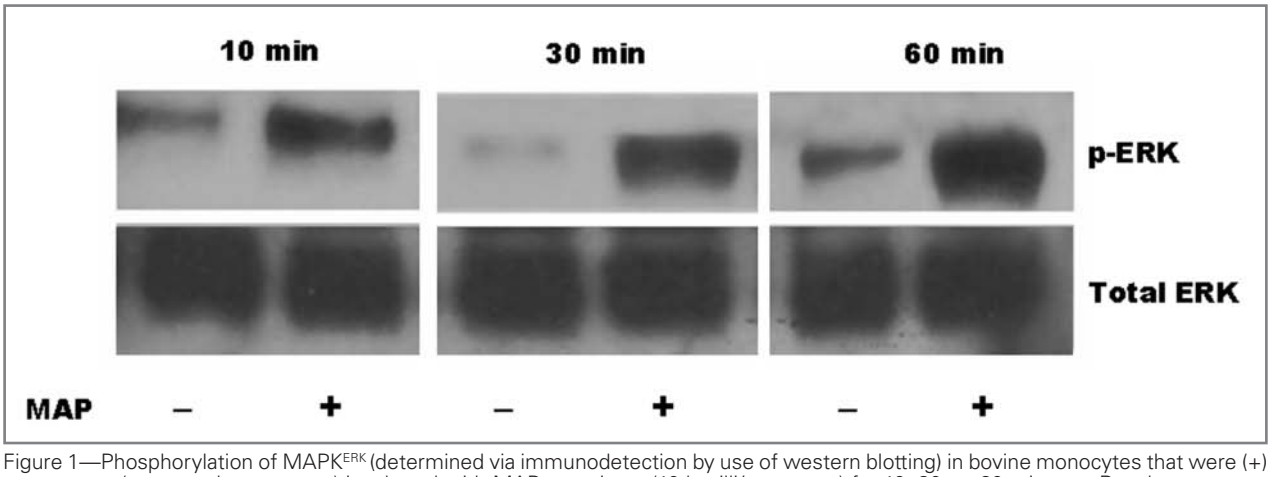
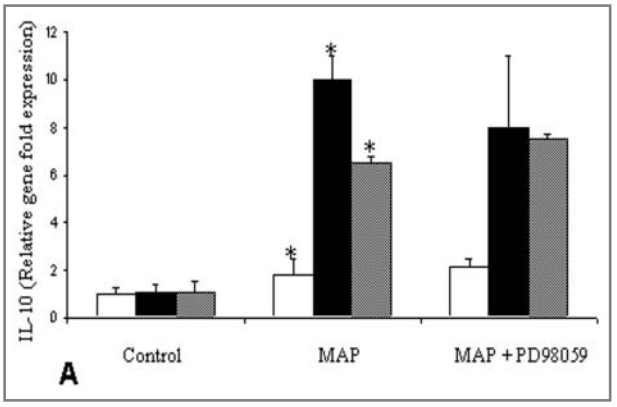
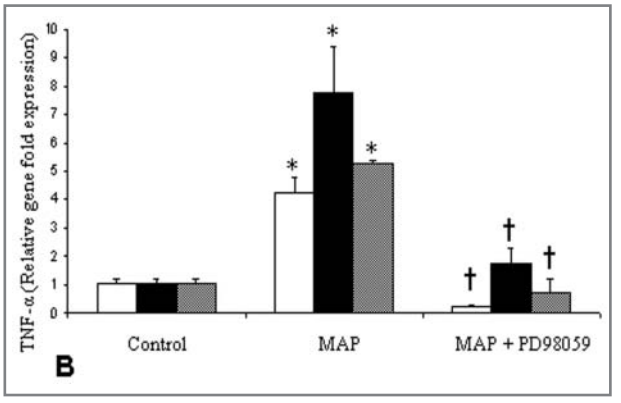


Figure 1—Phosphorylation of MAPK^{ERK} (determined via immunodetection by use of western blotting) in bovine monocytes that were (+) or were not (– ; control monocytes) incubated with MAP organisms (10 bacilli/monocyte) for 10, 30, or 60 minutes. Results represent phosphorylated ERK (p-ERK) and total ERK; similar data were obtained in 3 independent experiments.





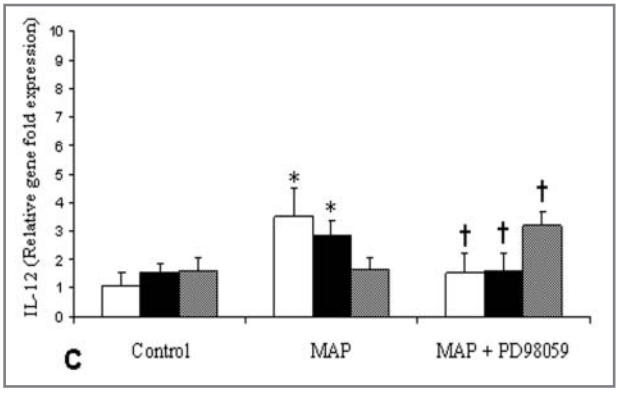


Figure 2—Mean ± SD relative gene fold expression of IL-10 (A), TNF-α (B), and IL-12 (C) mRNA by bovine monocytes that were or were not (control monocytes) incubated with MAP organisms (10 bacilli/monocyte) with or without PD98059 pretreatment for 2 (white bars), 6 (black bars), or 24 (gray bars) hours. Treatment with PD98059 (1 hour's duration) was performed before addition of MAP organisms to culture plates. Glyceraldehyde-3-phosphate dehydrogenase was used to adjust (normalize) the results. *Within a treatment group, value was significantly ($P < 0.05$) different from the value for control monocytes. †Value was significantly ($P < 0.05$) different from the value for monocytes exposed to MAP organisms alone for the same period of time.

Table 1—Effects of $MAPK^{ERK}$ inhibitor PD98059 on aspects of the antimicrobial activity of bovine monocytes exposed to MAP organisms.

		Incubation conditions	
Variable	**Incubation time (h)**	**MAP**	**MAP and PD98059**
Ingestion of MAP organisms (% of monocytes)	2	90 ± 3	92 ± 4
Killing of MAP organisms (% of dead organisms)	72	15 ± 3	22 ± 3
Phagosome acidification (colocalization coefficient)*	6	0.7 ± 0.2	0.8 ± 4
Nitrite (μM)	6	0.6 ± 0.15	0.7 ± 0.2
	24	3.5 ± 0.8	3.2 ± 0.5
Apoptosis (% of monocytes)	24	26 ± 3	27 ± 3

Three experiments were performed for each variable; results are reported as mean ± SD.
*Colocalization coefficient was defined as the density of red fluorescence divided by the density of green fluorescence.

vs 1.0). This increase persisted at 30 (3; control value, 1.0) and 60 minutes (2.6; control value, 1.4). Antibodies against phosphorylated and total $MAPK^{ERK}$ detected only 1 isoform of $MAPK^{ERK1/2}$ (approx 40 kd).

Effect of the $MAPK^{ERK}$ pathway on IL-10, TNF-α, and IL-12 mRNA expression—Bovine monocytes were pretreated with PD98059 (3 μM) for 1 hour at 37°C before addition of MAP organisms. Treatment with PD98059 alone had no effect on cytokine expression by monocytes (data not shown). Subsequently, monocytes were exposed to MAP organisms for 2, 6, or 24 hours and expression of IL-10, IL-12, and TNF-α mRNAs was analyzed by real-time PCR assay. Compared with control monocytes, MAP-exposed monocytes had greater expression of IL-10 at 2, 6, and 24 hours (**Figure 2**). Pretreatment with PD98059 did not affect IL-10 mRNA expression in monocytes at any time point after exposure to MAP organisms. Monocytes incubated with MAP organisms had greater expression of TNF-α at all time points. Monocytes incubated with MAP organisms and treated with PD98059 had lower expression of TNF-α at 2, 6, and 24 hours, compared with monocytes incubated with MAP organisms alone. The expression of IL-12 was greater at 2 and 6 hours in MAP-exposed monocytes than control monocytes. Compared with monocytes incubated with MAP organisms alone, monocytes incubated with MAP organisms and PD98059 had lower expression of IL-12 mRNA at 2 and 6 hours; however, IL-12 mRNA expression was greater in monocytes incubated with MAP organisms and PD98059 at 24 hours.

Role of $MAPK^{ERK}$ in the antimicrobial activity of bovine monocytes—The role of $MAPK^{ERK}$ in antimicrobial activity was assessed by evaluation of the effects of PD98059 on monocyte activities such as phagosome acidification, killing of MAP organisms, NO produc-

tion, and apoptosis. The PD98059 inhibitor alone had no effect on organism phagocytosis or monocyte viability (**Table 1**). Treatment of monocytes exposed to MAP organisms with PD98059 had no significant effect on phagosome acidification, killing of MAP organisms, apoptosis, or NO production at any time point, compared with findings in untreated cells.

Discussion

The antimicrobial response of mononuclear phagocytes to MAP organisms depends on the signals initiated during phagocytosis.[7,10,17-19] In the present study, involvement of the $MAPK^{ERK}$ pathway in the development of paratuberculosis was analyzed by the use of PD98059, which is a selective inhibitor of MEK1 and a dual-specificity kinase that activates $MAPK^{ERK}$ via phosphorylation of threonine and serine residues.[20] Our results indicated that MAP-induced TNF-α and IL-12 expression in bovine monocytes was significantly reduced in the presence of PD98059. However, inhibition of the $MAPK^{ERK}$ pathway had no effect on monocyte IL-10 mRNA expression or on several measures of antimicrobial activity.

Results of previous studies[21-23] support distinct roles of the 3 major MAPKs in cytokine expression associated with mycobacterial infection. The role of $MAPK^{ERK}$ has been studied[21] in human monocyte-derived macrophages infected with *M avium* subsp *avium* or *Mycobacterium tuberculosis*; as in the present study, $MAPK^{ERK}$ regulated TNF-α mRNA expression but did not regulate IL-10 mRNA expression.[21] In a previous study[22] of human monocyte-derived macrophages infected with *M tuberculosis*, addition of PD98059 resulted in an increase in IL-12 expression, which indicated that $MAPK^{ERK}$ played a negative role in regulating IL-12 expression. In the present study, addition of PD98059 initially resulted in a decrease in IL-12 expression with an increase in expression at 24 hours after exposure of monocytes to MAP organisms. These data have indicated that the role of $MAPK^{ERK}$ in IL-12 expression by MAP-exposed bovine monocytes is complex. The $MAPK^{JNK}$ pathway also appears to increase TNF-α expression in MAP-exposed bovine monocytes.[11] However, unlike $MAPK^{ERK}$, $MAPK^{JNK}$ decreases IL-12 expression in bovine monocytes at all time points after exposure to MAP organisms.[11]

Unlike $MAPK^{ERK}$ and $MAPK^{JNK}$ pathways, the $MAPK^{p38}$ pathway stimulates IL-10 expression in mycobacteria-infected mononuclear phagocytes but has little effect on TNF-α expression.[10,21] The $MAPK^{p38}$ pathway also suppresses IL-12 expression.[10] Because IL-10 is a major anti-inflammatory cytokine, its early expression could blunt the antimicrobial response of macrophages to MAP organisms and contribute to organism survival. Results of previous studies[10,14] support a key role of IL-10 in intracellular survival of MAP organisms. Treatment of bovine monocyte-derived macrophages with anti–IL-10 antibodies increases the capacity of those cells to acidify phagosomes and enables them to kill MAP organisms.[14] Taken together, these data indicate that each MAPK pathway plays a distinct role in cytokine expression associated with mycobacterial infection of mononuclear phagocytes.

Relatively little is known about the role of the $MAPK^{ERK}$ pathway in antimicrobial activity. In 1 study,[24] inhibition of growth of a less virulent morphotype of *M avium* subsp *avium* was partially dependent on expression of $MAPK^{ERK}$ by murine macrophages. In the present study, inhibition of the $MAPK^{ERK}$ pathway did not result in significant differences in multiple measures of antimicrobial activity in MAP-exposed bovine monocytes. This is surprising because we would expect that the decrease in TNF-α expression induced by PD98059 would decrease monocyte nitric oxide production or have inhibitory effects on apoptosis. However, several other TNF-α–independent factors (eg, nuclear factor-κB and caspases) are involved in regulating these processes.[6,8,16] Unlike $MAPK^{ERK}$, the $MAPK^{p38}$ pathway has a major effect on survival of MAP organisms in bovine monocytes.[10] Addition of a specific inhibitor of $MAPK^{p38}$ before exposure of monocytes to the mycobacteria results in increased phagosome acidification and organism killing. Therefore, the $MAPK^{p38}$ pathway but not the $MAPK^{ERK}$ pathway appears to have a major effect on antimycobacterial activity within bovine monocytes.

Results of the present study have indicated that $MAPK^{ERK}$ is activated on interaction of monocytes with MAP organisms and that this activation initiates monocyte TNF-α and IL-12 mRNA expression; however, our data failed to support an important role of this kinase in the antimicrobial activities of monocytes including NO production, apoptosis, phagosome acidification, or organism killing. We have concluded, therefore, that the $MAPK^{ERK}$ pathway may be important in initiation of proinflammatory and proimmune responses to MAP infection in cattle.

a. OADC, Difco Labs, Detroit, Mich.
b. Sigma, St Louis, Mo.
c. Allied Monitor Inc, Fayette, Mo.
d. Calbiochem, LaJolla, Calif.
e. RNeasy Kit, Qiagen, Valencia, Calif.
f. Cell lysis buffer, Cell Signaling, Beverly, Mass.
g. Pierce, Rockford, Ill.
h. Clone 9102, Cell Signaling, Beverly, Mass.
i. Clone 4377, Cell Signaling, Beverly, Mass.
j. DND-Free, Calbiochem, LaJolla, Calif.
k. Applied Biosystems, Foster City, Calif.
l. BackLight kit, Invitrogen, Carlsbad, Calif.
m. E800, Nikon Inc, Melville, NY.
n. Hoechst 33342, Molecular Probes, Eugene, Ore.

Appendix

Real-time PCR primers used to determine expressions of cytokine genes in bovine monocytes exposed to MAP organisms.

Gene	Primer
TNF-α	Forward 5′–TCAAACACTCAGGTCCTCTTCTCA–3′
	Reverse 5′–GTCGGCTACAACGTGGGCTACC–3′
IL-12 (p40)	Forward 5′–TCGGCAGGTGGAGGTCA–3′
	Reverse 5′–ACACAAAACGTCAGGGAGAAGTAG–3′
IL-10	Forward 5′–CGGCTGCGGCGCTGTCATC–3′
	Reverse 5′–TCACCTTCTCCACCGCCTTGCTCT–3′
GAPDH	Forward 5′–GAAACCTGCCAAGTATTGATGAGAT–3′
	Reverse 5′–TGTAGCCTAGAATGCCCTTGAGAG–3′

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

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