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Gene expression profiling of monocyte-derived macrophages following infection with *Mycobacterium avium* subspecies *avium* and *Mycobacterium avium* subspecies *paratuberculosis*

Judith T. Murphy, Sandra Sommer, Edward A. Kabara, Nitin Verman, Michael A. Kuelbs, Peter Saama, Robert Halgren, and Paul M. Coussens

Center for Animal Functional Genomics and Department of Animal Science, Michigan State University, East Lansing, Michigan

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*Mycobacterium avium* subspecies *avium* and *Mycobacterium avium* subspecies *paratuberculosis* represent two closely related intracellular bacteria with vastly different associated pathologies. MAA can cause severe respiratory infections in immune compromised humans but is nonpathogenic in ruminants and is more readily controlled by the bovine immune system than MAP. MAP causes a fatal wasting syndrome in ruminants, typified by granulomatous enteritis localized in the small intestine. MAP has also been cited as a potential cause of human Crohn’s disease. We used a bovine immune-specific microarray (BOTL-5) to compare the response of mature bovine monocyte-derived macrophages (MDM cells) to MAA and MAP. Statistical analysis of microarray data revealed 21 genes not appreciably expressed in resting MDM cells that were activated following infection with either MAA or MAP. Further analysis revealed 144 genes differentially expressed in MDM cells following infection with MAA and 99 genes differentially expressed following infection with MAP. Of these genes, 37 were affected by both types of mycobacteria, with three being affected in opposite directions. Over 41% of the differentially expressed genes in MAA and MAP infected MDM cells were members of, regulated by, or regulators of the MAPK pathways. Expression of selected genes was validated by quantitative real-time reverse transcriptase PCR and in several key genes (i.e., IL-2 receptor, tissue inhibitor of matrix metalloproteinases-1, and Fas-ligand) MAA was found to be a stronger activating factor than MAP. These gene expression patterns were correlated with prolonged activation of p38 MAPK and ERK1/2 by MAA, relative to MAP.

cDNA microarray; macrophage; mycobacteria

Johne’s disease in cattle is due to prolonged survival of infected cells, to enhanced migration of monocytes to sites of infection, or to both. Gene expression profiling studies suggest that IL-1α and the antiapoptotic protein TNF receptor-associated factor (TRAF)1 are highly expressed in MAP-induced lesions and these factors may contribute to macrophage survival (1).

To gain insight into differences in how macrophage cells respond to or are affected by MAA and MAP, we employed an in vitro monocyte-derived macrophage (MDM) culture system. For these studies, we utilized primary bovine macrophages, since cattle are a natural host for both MAP and MAA. Gene expression profiling of infected and control MDM cells was accomplished using a cDNA microarray focused on bovine orthologs of immune response genes, as well as expressed sequence tag (EST) sequences from a bovine total leukocyte library (BOTL-5). Our results demonstrate that both MAA and MAP have profound effects on MDM gene expression, that these two closely related organisms affect expression of many genes in a similar manner, but that there are important differences that may be related to persistence and virulence.

MATERIALS AND METHODS

Bacterial cultures. MAA and MAP were obtained from the American Type Culture Collection (ATCC #35716 and ATCC #19698, respectively) and grown at 37°C in Middlebrooks 7H9 media with 10% Middlebrooks OADC enrichment, and, for MAP only, Mycobactin J (Allied Monitor, Lexana, KS) was added at 2 mg/l for 12–16 wk. MAA and MAP were serially diluted and counted on a bacterial hemocytometer. Infection of macrophage cells, bacterial suspensions were placed in a sonicating water bath for 10 min and vigorously vortexed to disperse clumps and diluted in phosphate-buffered saline (PBS).

Experimental animals. Healthy control cattle ranging in age from 12 to 48 mo were used as a source of MDM cells in this study (n of 6). All cattle were of the Holstein breed and housed at the Michigan State University Dairy farm. The immune status of control cattle with regard to infection with MAA had been monitored by serum enzyme-linked immunosorbent assay (ELISA) and periodic IFN-γ testing (Bovigam; Biocor Animal Health, Omaha, NE) before initiation of experiments. Fetal culture testing by a US Department of Agriculture-approved testing laboratory (Michigan State University Diagnostic Center for Animal and Population Health, East Lansing, MI) was conducted to confirm negative infection status. All animals were serum ELISA negative, IFN-γ negative, and fecal culture negative.

Preparation of bovine MDM. For isolation of primary bovine MDM, blood was collected from the tail vein of healthy donor cattle using 8-ml vacutainers (BD, Franklin Lakes, NJ) containing 1.5 ml of acid citrate dextrose as anticoagulant. Blood was centrifuged for 20 min at 600 g at room temperature. Plasma was removed. Buffy coat cells were transferred to 50-ml conical tubes containing 10 ml of room temperature sterile Percoll (1.084 g/cm³; Sigma, St. Louis, MO) layered under 30 ml of sterile PBS and centrifuged for 40 min at 400 g at room temperature. Mononuclear cells were collected from the PBS-Percoll interface and washed one time with cold PBS. For preparation of bovine MDM, isolated mononuclear cells were plated at 1.0 × 10⁶ cells in T-25 culture flasks and incubated for 2 h in RPMI 1640 tissue culture medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO) at 39°C and 5% CO₂ to allow monocytes to adhere.

After 2 h of incubation, nonadherent cells were removed by washing three times with warm (39°C) PBS. Adherent monocyte cells were allowed to differentiate in culture for 5–7 days in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum at 39°C and 5% CO₂. Following differentiation, MDM cells were checked for morphology under a light microscope. The final yield of MDM cells was ~1 × 10⁷ cells/flask. Before various treatments [MAA, MAP, or uninfected (Nil)], MDM cells were washed three times with warm PBS, and all treatments were conducted in RPMI 1640 tissue culture medium without phenol red supplemented with 10% fetal bovine serum.

Infection of bovine MDM with MAA and MAP. Bovine MDM from six healthy control cattle were cultured in RPMI 1640 tissue culture medium without phenol red supplemented with 10% fetal bovine serum and, on day 7 of culture, were infected with MAA and MAP at a multiplicity of infection (MOI) 10:1 (10 bacilli/macrophage) for 24 h. Previous studies demonstrated that an MOI of 10:1 produced optimal uptake of MAP (40–60% of cells infected), and increasing the number of bacilli beyond this had little effect on the percentage of infected cells (data not shown). The 24-h time point was chosen to allow ingested mycobacteria time to express genes and proteins following adaptation to the intracellular environment and to allow any mycobacterial proteins to exert their effects on macrophage gene expression and phenotypes. Cells were lysed in a commercial RNA extraction buffer (Versagene Kit; Gentra Systems, Minneapolis, MN), and RNA was extracted essentially as recommended by the manufacturer. RNA quality was assessed using an Agilent Bioanalyzer 2100 and the RNA NanoChop essentially as recommended by the manufacturer (Agilent Technologies, Palo Alto, CA) and on a nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Preparation of labeled cDNA and microarray hybridization. To evaluate gene expression profiles of infected and control MDMs, total RNA (8 µg) from each treatment was used as template in reverse transcription reactions (Atlas Powerscript labeling system; BD Biosciences, Alameda, CA) in which oligo(dT)15–18 was used as primer. In the Atlas Powerscript system, cDNA is prepared with a randomly incorporated amino-modified dUTP.

Following first-strand synthesis, cDNAs from all treatment groups (Nil, MAA, and MAP) were labeled using N-hydroxysuccinimide-derivatized Cy3 and Cy5 dyes (Amer sham Pharmacia, Piscataway, NJ). A loop design was used within cow, such that the Nil-treated sample (Cy3) was directly compared with the MAP-infected sample (Cy5), the MAP-infected sample (Cy3) was directly compared with the MAA sample (Cy5) and the MAA sample was directly compared with the Nil-treated sample (Cy5) for MDM cells from three cows. The design was reversed in terms of dye labeling for MDM cells from the other three cows, to offset potential dye bias. Labeled cDNAs were purified to remove unincorporated dyes using cDNA labeling purification modules (Invitrogen, Carlsbad, CA), as previously described (14–16). Differentially labeled samples (infected vs. control) were then combined and concentrated to 10 µl by using Microcon 30 spin concentrators (Millipore, Bedford, MA). Microarray hybridization was performed by addition of concentrated Cy3-Cy5-labeled probe cDNAs to 100 µl of SlideHyb-3 (Ambion, Alameda, CA).

Microarray hybridizations were conducted for 18 h in a commercial microarray hybridization station by using a step-down hybridization protocol (GeneTAC; Genomics Solutions, Ann Arbor, MI) as described previously (15, 17, 19). BOTL-5 bovine cDNA microarrays from the Center for Animal Functional Genomics (CAFG) at Michigan State University were employed in this study. These microarrays have been described previously (15, 17) and contain a combination of ~750 EST clones from a bovine total leukocyte cDNA library and 750 PCR amplicons representing known immune response genes, all spotted in duplicate. The full list of genes represented on BOTL-5 microarrays as well as other annotation can be found at www.cafg.msu.edu under the Search Libraries section, selecting to browse clones by library. Complete annotation is found under the GeneLinks section of this same page.

Following hybridization, cDNA microarrays were washed in the hybridization station, rinsed once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and once in (dd)-distilled H₂O and
Finally dried by centrifugation in a cushioned 50-ml conical centrifuge tube. This process yielded BOTL-5 cDNA microarrays, which allowed direct comparison of MDM gene expression between all three treatment groups.

Final hybridized microarrays were scanned by using a GeneTAC LS IV microarray scanner and GeneTAC LS software (Genomic Solutions). Gene TAC analyzer software was then used to process microarray images, find spots, integrate robot-spotting files with the microarray image, and finally to create reports of raw total spot intensities for both dyes. LOWESS-normalized microarray data used in this report can be found under the links section of the CAFG website (www.cafg.msu.edu).

Microarray data analysis. Raw total intensity values for each spot on the microarray were converted to comma separated values files, transferred to Excel spreadsheets, and prepared for LOWESS normalization using PROC LOESS from the statistical software package SAS (11, 40, 41). Success of LOWESS normalization was monitored by plotting M (logCy3 − logCy5) vs. A [(logCy3 + logCy5)/2] for each microarray (49). LOWESS-normalized microarray data were subsequently processed essentially as described (15−17). Initially, the two gene replicate spots on each BOTL-5 microarray were combined to yield a mean LOWESS-normalized intensity value for each dye (Cy3 and Cy5) and each gene. Mean LOWESS-normalized values (log2) were back transformed, and the median negative value for each dye within array was subtracted to account for background intensities. Background corrected values were retransformed (natural log, ln) and used to calculate ln difference values (Nil vs. MAA, MAA vs. MAP, and MAP vs. Nil) for each gene represented on the BOTL-5 microarray. This process identifies genes expressed at levels below background in each sample, since negative values cannot be retransformed. Genes that were scored as below background in four or more of the six biological replicates in this study were scored as not expressed.

In subsequent analyses to identify genes differentially expressed between the treatment groups, we employed a mixed-models procedure essentially as described previously (15, 48). Briefly, LOWESS-adjusted log intensities were analyzed statistically using a mixed-model approach consisting of two steps. The first step involved array-specific spatial variability normalization and the second step, gene-specific analyses to test effects of infection group (Nil, MAA, or MAA) on expression profiles for individual genes. The normalization model in the first step included the fixed effects of dye, group, and their interaction, as well as the random effects of array, animal within group, and patch within array. The second step of the statistical analysis consisted of gene-specific models for estimated residuals obtained from the normalization approach discussed above. Split plot models were considered here, having animals as plot (each in one of the groups) and dyes as subplots (with each of the infections). Models included gene-specific fixed effects of dye, infection group, and their interaction, and random effects of animal within infection group, patch within array, and spots within patches. These analyses were computed by using the MIXED procedure of SAS. Benjamini-Hochberg false discovery rate adjustments for the significance of the difference between group means were performed using the MULTTEST procedure of SAS. Final data were then filtered to highlight genes that were significantly differentially expressed (P < 0.05) in the comparisons of MAA vs. Nil, MAP vs. Nil, and MAP vs. MAA.

RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction. RNA was extracted from MDM cell samples as described above. All RNA samples were treated with RNase-free DNase I (Promega, Madison, WI). Quality and quantity of extracted total RNA was estimated using an Agilent Bioanalyzer 2100 and RNA nanochip (Agilent, Foster City, CA).

Quantitative real-time reverse transcriptase polymerase chain reactions (Q-RT-PCR) were performed in an Applied Biosystems 7000 DNA sequence detection system (Perkin Elmer, Foster City, CA), essentially as described previously (18, 19). Briefly, total RNA extracted from MDM cell samples was converted into first-strand cDNA by adding 2 μg of total RNA to a 12-μl reaction containing 10 mM oligo(dT)15−18 primer. Following a 5-min incubation at 70°C, the reaction was quick-chilled to 20°C and adjusted by addition of 4 μl of a 5× buffer supplied by the RT manufacturer (final reagent concentrations were: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl2), 1 mM dNTPs, 200 units of Superscript II RNase H− Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA), and a final concentration of 10 mM DTT in a total reaction volume of 20 μl. The RT reaction was allowed to progress at 42°C for 60 min, heated to 70°C for 15 min, and cooled to 37°C before the addition of 2 units of DNase-Free RNaseH (Invitrogen Life Technologies). Incubation at 37°C continued for 20 min in the presence of RNaseH to remove original RNA templates. RNaseH was subsequently inactivated by heating at 70°C for 10 min. First-strand cDNAs were purified by extraction with Quick-Clean Resin (BD Biosciences, Alameda, CA) and precipitation in ethanol. Final cDNA pellets were suspended in 52 μl of RNase-Free ddH2O. Concentration of cDNA in each sample was determined by Nanodrop 1000 spectrometry (Nanodrop Technologies). Gene TAC analyzer software was then used to calculate ln difference values (Nil vs. MAA, MAA vs. MAP, and MAP vs. Nil) for each gene represented on the BOTL-5 microarray.

Q-RT-PCR data were analyzed by the 2−ΔΔCt method as described (19, 33). To assess the effect of MAA or MAP infection of MDM cells, β-actin served as the control gene (calculation of ΔCt) and uninfected cells (Nil) served as the calibrator (calculation of ΔΔCt). Western blot analysis of MAP kinase activation. MDM cells were isolated and cultured as described previously. On day 7 of culture, MDM cells were infected with MAP or MAA at an MOI of 10:1 and allowed to interact with bacteria for 0, 15, 30, or 60 min postinfection. Cells were harvested using an SDS-PAGE sample buffer and proteins subjected to SDS-PAGE in 12.5% polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes and probed with primary antibodies (all from Cell Signaling, Danvers, MA) specific for total p38 (9212), phospho-p38 (9215), total ERK1/2 (9102), or phospho-ERK1/2 (4377). All primary antibodies were used at a dilution of 1:500 in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST). Following a 1-h incubation with diluted primary antibody, membranes were washed three times in TBST. Secondary anti-rabbit antibody (sc2313; Santa Cruz Biotechnology, Santa Cruz, CA) conjugated to horseradish peroxidase and a commercial chemiluminescent substrate (Pierce, Rockford, IL) were used to detect primary antibody binding.

RESULTS

Activation of quiescent genes by MAA and MAP. In total, we performed 18 microarray analyses, comparing Nil-, MAA-, and MAP-treated MDM cells with a loop design, within cow for MDM cells from six healthy donors. In our first analysis following normalization of microarray data as described in MATERIALS AND METHODS, we sought to identify genes that were expressed at very weak levels or not at all in resting (Nil) MDM cells but were activated upon infection with either MAA

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by adding 2 μg of total RNA to a 12-μl reaction containing 10 mM oligo(dT)15−18 primer. Following a 5-min incubation at 70°C, the reaction was quick-chilled to 20°C and adjusted by addition of 4 μl of a 5× buffer supplied by the RT manufacturer (final reagent concentrations were: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl2), 1 mM dNTPs, 200 units of Superscript II RNase H− Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA), and a final concentration of 10 mM DTT in a total reaction volume of 20 μl. The RT reaction was allowed to progress at 42°C for 60 min, heated to 70°C for 15 min, and cooled to 37°C before the addition of 2 units of DNase-Free RNaseH (Invitrogen Life Technologies). Incubation at 37°C continued for 20 min in the presence of RNaseH to remove original RNA templates. RNaseH was subsequently inactivated by heating at 70°C for 10 min. First-strand cDNAs were purified by extraction with Quick-Clean Resin (BD Biosciences, Alameda, CA) and precipitation in ethanol. Final cDNA pellets were suspended in 52 μl of RNase-Free ddH2O. Concentration of cDNA in each sample was determined by Nanodrop 1000 spectrometry (Nanodrop Technologies). Gene TAC analyzer software was then used to calculate ln difference values (Nil vs. MAA, MAA vs. MAP, and MAP vs. Nil) for each gene represented on the BOTL-5 microarray.

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or MAP. To accomplish this, LOWESS-normalized values were back transformed, and the median blank value within each dye on each array was subtracted from the values for every gene. In this way, genes that were not expressed above background levels would return a negative result that could not be retransformed. In order for a gene to be considered as not expressed in Nil-treated MDM cells, but activated upon infection, we used a cutoff of at least four of six data points being positive in the infected cells and no more than two of six in the Nil-treated sample being positive. In total, we identified 21 such genes in the MAA vs. Nil samples and in the MAP vs. Nil samples (Table 1). Of note were genes encoding IL-1 receptor antagonist, TWEAK, IL-6, IL-7 receptor, IL-12 receptor β2 and IL-1 receptor antagonist

Table 1. Genes not expressed in Nil-treated samples from MAP vs. Nil microarrays (n = 6)

<table>
<thead>
<tr>
<th>Gene Encoding</th>
<th>Nil</th>
<th>MAP</th>
<th>MAA</th>
<th>MAP/MAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP7 - bone morphogenetic protein 7</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>-3.33</td>
</tr>
<tr>
<td>BOTL0100003XB08R</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>1.33</td>
</tr>
<tr>
<td>CD4 antigen</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>2.27</td>
</tr>
<tr>
<td>CD8 antigen</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>3.54</td>
</tr>
<tr>
<td>CNTFR - ciliaryneurotrophic factor receptor</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>1.06</td>
</tr>
<tr>
<td>EphB1</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>1.05</td>
</tr>
<tr>
<td>Ephrin-A1</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>4.12</td>
</tr>
<tr>
<td>Ephrin-A5</td>
<td>not expressed</td>
<td>expressed</td>
<td>not expressed</td>
<td>2.45</td>
</tr>
<tr>
<td>CDK2 - cyclin-dependent kinase</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>-8.9</td>
</tr>
<tr>
<td>IL-1-RA - IL-1 receptor antagonist</td>
<td>not expressed</td>
<td>expressed</td>
<td>not expressed</td>
<td>1.45</td>
</tr>
<tr>
<td>IL-12R β2 - IL-12 receptor β2</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>1.75</td>
</tr>
<tr>
<td>IL-6</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>-2.26</td>
</tr>
<tr>
<td>IL-7 receptor-α</td>
<td>not expressed</td>
<td>expressed</td>
<td>not expressed</td>
<td>1.60</td>
</tr>
<tr>
<td>INTB5 - integrin β-5</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>2.08</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>1.82</td>
</tr>
<tr>
<td>SLAM - signaling lymphocytic activation molecule</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>-12.9</td>
</tr>
<tr>
<td>Somatic cytochrome</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>-1.33</td>
</tr>
<tr>
<td>STK-1 - growth factor receptor tyrosine kinase</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>1.83</td>
</tr>
<tr>
<td>TANK - TRAF family member-associated NF-κB activator</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>4.80</td>
</tr>
<tr>
<td>TWEAK</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>1.96</td>
</tr>
<tr>
<td>VCAM-1 vascular cell adhesion molecule-1</td>
<td>not expressed</td>
<td>expressed</td>
<td>expressed</td>
<td>1.29</td>
</tr>
</tbody>
</table>

Nil, untreated; MAP, Mycobacterium avium subspecies paratuberculosis; MAA, Mycobacterium avium subspecies avium.

merged and examined for similarities and differences. In total, we identified 144 genes that were significantly differentially expressed in MAA-infected cells relative to Nil-treated cells (P < 0.05), 99 genes that were significantly differentially expressed in MAP-infected MDM cells relative to Nil-treated cells (P < 0.05), and 124 genes that were significantly differentially expressed in MDM cells infected with MAA vs. MAP. The intersection of these gene lists indicated that 37 genes were common within the comparisons between MAA and MAP and Nil and MAP and Nil. Of these, only three genes were affected in opposite directions by the two strains of mycobacteria (Fig. 1). This rather large set of shared effects is consistent with the genetic similarities between MAA and MAP (>98% [3]). In addition, the larger group of affected genes in MAA-infected MDM cells relative to MAP-infected cells is consistent with the generally accepted view that MAA is a stronger stimulator of MDM cells.

The relative expression of these genes was further evaluated in the direct comparison of MAP-infected MDM cells to MAA-infected cells (Table 1). In many cases, MAP and MAA affected expression to a similar extent (fold change <2.0), but there were notable exceptions. This analysis predicted that the genes encoding BMP7, CDK2, IL-6, and SLAM would be expressed at significantly lower levels in MAP-infected MDM cells than in MAA-infected cells. Importantly, these proteins are all members of or are regulated by the three major MAPK pathways.

Similarities and Differences in MAA- and MAP-infected MDM cells. We next wished to identify genes that were similarly expressed in MDM cells following infection with MAA and MAP, as well as genes that were differentially expressed in the two infection groups. To accomplish this, we performed a two-step mixed models analysis as described previously (15, 48), and in MATERIALS AND METHODS. Separate lists of differentially expressed genes were created for MAA vs. Nil, MAP vs. Nil, and MAA vs. MAP many genes appeared on more than one list, as would be expected. These lists were
of macrophage cells than MAP. This distinction is further accentuated by the fact that in the MAA vs. Nil gene set, 77 genes were expressed at higher levels in MAA-treated cells than in Nil-treated cells, while in the MAP vs. Nil, only 43 genes were expressed at higher levels in MAP-treated cells relative to Nil-treated cells.

Of particular interest, following both automated and manual annotation of the significantly differentially expressed genes, >41% were found to be members of, regulated by, or to regulate the MAPK pathway. In contrast, of the 630 non-redundant genes on the BOTL-5 microarray with defined product names (i.e., those encoding known proteins), 168 (26%) have possible connections to the MAPK pathway.

Q-RT-PCR analysis of selected differentially expressed genes. Based on our initial microarray results, we selected a number of genes for which we were interested in gaining further insight and validation. Our selection criteria were based in part on those genes that were common between MAA- and MAP-infected MDM cells, those that were not significantly expressed in Nil-treated cells but were efficiently expressed in MAA- and MAP-treated cells, and several genes known to be regulated by members of the MAPK pathway. Thus, we selected genes encoding Fas ligand (FasL), tissue inhibitor of matrix metalloproteinases-1 (TIMP1), BCL10, BCL2, signal transducer and activator of transcription-6, fibroblast growth factor related proteins, including FGF and the SRF. Gene-specific primers were prepared for all selected genes and Q-RT-PCR conducted as described previously (18, 19) and in MATERIALS AND METHODS. In all cases, expression in MAA- and MAP-infected MDM cells was presented relative to Nil-treated cells within cow and MDM cells from four to six distinct healthy cows were used as biological replicates. As shown in Fig. 2, MAA and MAP have profound effects on expression of the apoptosis-related genes FasL and BCL10 in MDM cells. MAA induced FasL gene expression >30-fold relative to Nil-treated cells, while MAP induced FasL gene expression significantly more weakly, at 15-fold, but still significantly more than in Nil-treated cells. MAA and MAP induced BCL10 gene expression to approximately equivalent levels, approximately three- to fourfold over that observed in Nil-treated cells (Fig. 2). In contrast, both mycobacteria reduced expression of the BCL2 gene by approximately one-third. Although the RIP kinase was suggested to be differentially expressed in MAA- and MAP-infected cells by microarray analysis, this could not be confirmed by Q-RT-PCR (Fig. 2).

MAA and MAP affected expression of several genes encoding growth factor related proteins, including FGF and the SRF. These changes were validated by Q-RT-PCR (Fig. 3). For FGF gene expression, MAA tended to be a stronger stimulus than MAP, but this trend was not statistically significant (P > 0.05). SRF gene expression was of interest as this was the only validated case where MAP induced significantly higher (P < 0.05) expression than MAA, ~5.5-fold for MAP compared with less than twofold for MAA (Fig. 3). Expression of genes encoding proteins involved in the proinflammatory response, IL-6, iNOS, IL-2 receptor (CD25), and TIMP1 was also evaluated by Q-RT-PCR (Fig. 4). For iNOS and IL-6 gene expression, there were no statistically significant differences between MAA and MAP. In contrast, expression of IL-2R and TIMP1 genes was significantly higher in cells infected with MAA than in either uninfected cells or cells infected with MAP (Fig. 4). For IL-2R and TIMP1, MAA was a much stronger (3- to 5-fold) inducer of proinflammatory gene expression than was MAP. The rather large upregulation of IL-6 gene expression by both MAA and MAP (15- to 30-fold) is consistent with other gene expression studies in primary human macrophage cells (7).

MAA and MAP both affect the MAPK pathways, but with important differences. Because many of the differentially expressed genes we observed in MAA- and MAP-infected MDM cells relative to uninfected cells were members of, regulated by, or regulators of the three major MAPK pathways, we next sought to examine the effects of MAA and MAP on activation of p38 MAPK and the extracellular receptor kinases (ERK1/2). Cross-reactive antibodies that recognize the Jun NH2-terminal

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Fig. 2. Expression of genes encoding apoptosis-related proteins in mature MDM cells following infection with MAA or MAP. Gene expression was assessed by quantitative real-time (Q) RT-PCR as described previously (18) using gene-specific primers and SYBR Green detection. Data were analyzed by the 2^(-dCt/dAct) method with β-actin as control gene and the mean Nil sample values for dCt as the calibrator, also as described previously (18, 33). In the plots shown, the bars represent means of MDM cultures from 4 different healthy donor cows. Error bars represent SE between the 4 biological replicates.
kinase (JNK) have not yet been identified, and thus this could not be included in the present study. Activation of p38 MAPK and ERK1/2 was analyzed using cross-reactive antibodies specific for total p38 and total ERK1/2 to monitor protein levels. A second set of antibodies specific for the phosphorylated forms of p38 and ERK1/2 was used to assess activation (phosphorylation) following infection of MDM cells with MAA or MAP, as described in MATERIALS AND METHODS. Infection of MDM cells with both MAA and MAP rapidly induced phosphorylation of p38 MAPK and ERK1/2. Within 15 min of application of MAA or MAP, readily detectable levels of both phosphorylated proteins were observed (Fig. 5, A and B). By 30 min postinfection, however, MDM cells infected with MAP contained significantly less phospho-p38 (p-p38) and phospho-ERK1/2 than cells infected with MAA (Fig. 5, A and B). Within 60 min postinfection, phospho-p38 induced by MAP had returned to levels indistinguishable from uninfected cells, while MAA-infected cells still displayed some phospho-p38 (Fig. 5A). At 60 min postinfection, levels of phospho-ERK1/2 (p-ERK1/2) had normalized in cells infected with MAP and MAA but were still higher than uninfected cells (Fig. 5B).

**DISCUSSION**

In this study, we sought to define similarities and differences in gene expression patterns in mature MDM cells following infection with MAA and MAP, two closely related mycobacteria that differ in their respective pathologies. Although MAA can be pathogenic in immune-compromised humans (24, 25), it is generally thought of as a nonpathogenic environmental microorganism (28). In cattle, infections with MAA are usually quickly resolved with a rapid and appropriate proinflammatory and cytotoxic immune response (20). In contrast, infection of ruminants with MAP leads to a long-term chronic infection characterized by granulomatous enteritis in the small intestine with numerous acid-fast staining macrophages. Eventually infections with MAP are fatal in ruminants, with survival periods highly dependent upon host species (10, 12, 30). Survival of MAP in macrophage cells is generally viewed as a key feature of this organism’s ability to survive and evade the host immune system (12). In addition, it has been suggested that MAP-infected macrophages may be defective in activation and signaling, contributing to the loss of appropriate Th1-like re-

**Fig. 3.** Expression of genes encoding the cell growth-related proteins, fibroblast growth factor (FGF) and serum response factor (SRF), in mature MDM cells following infection with MAA or MAP. Gene expression was assessed by Q-RT-PCR as described previously (18) using gene-specific primers and SYBR Green detection. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with β-actin as control gene and the mean Nil sample values for dCt as the calibrator, also as described previously (18, 33). In the plots shown, the bars represent means of MDM cultures from 4 different healthy donor cows. Error bars represent SE between the 4 biological replicates.

**Fig. 4.** Expression of genes encoding proteins related to the proinflammatory response in mature MDM cells following infection with MAA or MAP. Gene expression was assessed by Q-RT-PCR as described previously (18) using gene specific primers and SYBR Green detection. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with β-actin as control gene and the mean Nil sample values for dCt as the calibrator, also as described previously (18, 33). In the plots shown, the bars represent means of MDM cultures from 4 different healthy donor cows. Error bars represent SE between the 4 biological replicates.
study using mature bovine MDM cells (45). TNF-α gene expression was not appreciably enhanced in MAA- or MAP-infected MDM cells in the present study. These results are in conflict with previous investigations using human MDM cells and various strains of MAA (7). However, in both the present study and that performed by Weiss et al. (45), IL-6 gene expression was dramatically upregulated in mature bovine MDM cells following infection with either MAA or MAP. IL-6 is a critical proinflammatory cytokine, and stimulation of T cells in the presence of IL-6 can overcome blocks by regulatory T cells. Sustained production of IL-6 has been previously identified as important in the induction of protective immune responses to mycobacteria (2, 35). Although expression of TNF-α and IL-6 is generally linked together under a proinflammatory response pattern, it is clear that expression of these two cytokines is uncoupled in MDM responses to MAA and especially to MAP. Uncovering the molecular basis of this uncoupling could help explain how MAA and MAP survive in macrophage cells and how infection with these organisms affects the ability of macrophages to communicate with other immune cells. Importantly, both the IL-6 and TNF-α genes appear to be regulated via the MAPK pathway.

Other themes consistent between this study and previous analyses (47, 49) include enhanced expression of transforming growth factor-β in MDM cells infected with MAP relative to cells infected with MAA, upregulation of various H+ ATPases in MAP-infected cells relative to Nil-treated cells, changes in expression of various GTPases in infected cells, and decreased expression of genes encoding LAMPs in MAP-infected cells relative to MAA-infected cells. While at the present time, the effects of these differences in gene expression in terms of macrophage function are not clear, they do suggest exciting new avenues of research into the molecular interactions between mycobacteria and host macrophage cells. In addition, these results suggest that infection of macrophages with MAP or MAA may affect how these cells interact with other immune cells.

Another theme that appears to be emerging is that MAA is, in general, a more potent inducer of proinflammatory and other genes in MDM cells than MAP. In the present study, we demonstrated that MAA and MAP both activate expression of numerous genes in resting mature macrophage cells upon infection. However, MAA appeared to be a much stronger activator of most differentially expressed genes, with one notable exception (i.e., SRF, Fig. 3). Although iNOS gene expression tended to be slightly higher in MAP-infected MDM cells than in MAA-infected cells, this difference was not statistically significant (Fig. 4). Of note in this pattern from the present study was greater expression of FasL, CD25, and TIMP1 in MDM cells infected with MAA, relative to cells infected with MAA, upregulation of various H+ ATPases in MAP-infected cells relative to Nil-treated cells, changes in expression of various GTPases in infected cells, and decreased expression of genes encoding LAMPs in MAP-infected cells relative to MAA-infected cells. While at the present time, the effects of these differences in gene expression in terms of macrophage function are not clear, they do suggest exciting new avenues of research into the molecular interactions between mycobacteria and host macrophage cells. In addition, these results suggest that infection of macrophages with MAP or MAA may affect how these cells interact with other immune cells.

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with MAA or MAP alters macrophage susceptibility to external and internal apoptotic pathways.

Our finding that infection of mature MDM cells with MAA or MAP induced expression of the IL-2R (CD25; Fig. 4) was initially surprising as IL-2 is generally thought of as a T cell cytokine. While IL-2 is indeed produced primarily by activated T cells, recent evidence suggests that macrophages also express CD25 and that activation with IL-2 complements other signals, such as TNF-α and IL-12, in induction of IFN-γ gene expression and IFN-γ protein secretion (21, 39, 44). In this regard, it is especially interesting that MAA was a much more potent inducer of CD25 gene expression than MAP (Fig. 4), suggesting that IL-2 would have more profound effects on macrophages infected with MAA than similar cells infected with MAP. Previous studies have confirmed that macrophages treated with IFN-γ and IL-2 in combination are better able to control infections with intracellular pathogens than macrophages treated with either cytokine alone (21, 39, 44).

Finally, a high percentage of genes found to be differentially expressed in MDM cells infected with either MAA or MAP (41%) were found to be members of, to be regulated by, or to regulate one of the three main MAPK pathways (p38, ERK1/2, or JNK). This is consistent with previous studies in bovine and human MDM cells (7, 45). In particular, those genes showing a clear differential regulation by MAA compared with MAP in the current study, including IL-6, CD25, FasL, and TIMP1, are clear examples of genes encoding MAPK pathway associated genes. A recent study also suggested that the p38 MAPK pathway was important in macrophage response to MAP infection (42). It was of interest therefore to examine activation of the MAPK pathways in mature MDM cells following infection with MAA and MAP. Our results suggest that p38 MAPK and ERK1/2 are rapidly activated via phosphorylation in response to infection of MDM cells with either MAA or MAP. However, by 30 min postinfection, the amount of detectable p-p38 and p-ERK1/2 is considerably reduced in MAP-infected MDM cells, but not in MAA-infected MDM cells. This important difference in MAPK pathway activation may help explain the patterns of gene expression we have observed, with MAA eliciting a much stronger gene expression response than MAP, in most cases. Two important caveats to this is that while our microarray studies used cells that had been infected for 24 h, MAPK activation is typically observed within a few minutes of infection, although activation is usually cyclic. In addition, we were not able to identify antibodies to study activation of the JNK MAPK pathway in bovine MDM cells; a complete analysis will need to include this important aspect. Future studies using time courses and various MAPK inhibitors should also help to define the molecular basis of differential activation by MAA and MAP.

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