Host gene expression for *Mycobacterium avium* subsp. *paratuberculosis* infection in human THP-1 macrophages

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One sentence summary: Host gene expression for MAP infection.

**ABSTRACT**

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of Johne’s disease, which causes considerable economic loss in the dairy industry and has a possible relationship to Crohn’s disease (CD) in humans. As MAP has been detected in retail pasteurized milk samples, its transmission via milk is of concern. Despite its possible role in the etiology of CD, there have been few studies examining the interactions between MAP and human cells. In the current study, we applied Ingenuity Pathway Analysis to the transcription profiles generated from a murine model with MAP infection as part of a previously conducted study. Twenty-one genes were selected as potential host immune responses, compared with the transcriptional profiles in naturally MAP-infected cattle, and validated in MAP-infected human monocyte-derived macrophage THP-1 cells. Of these, the potential host responses included up-regulation of genes related to immune response (*CD14, S100A8, S100A9, LTF, HP and CHCIL3*), up-regulation of Th1-polarizing factor (*CCL4, CCL5, CXCL9 and CXCL10*), down-regulation of genes related to metabolism (*ELANE, IGF1, TCF7L2 and MPO*) and no significant response of other genes (*GADD45a, GPNMB, HMOX1, IFNG and NQO1*) in THP-1 cells infected with MAP.

**Keywords:** *Mycobacterium avium* subsp. *paratuberculosis*; host responses; THP-1

**INTRODUCTION**

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) causes Johne’s disease (JD), a chronic granulomatous enteropathy of ruminants such as cattle, sheep, bison and elk (Motiwala et al. 2006). Concerns have been raised about JD over economic losses in the dairy industry as well as the possible relationship to a chronic inflammatory bowel disease (IBD) in humans called Crohn’s disease (CD) (Thayer et al. 1984; Naser et al. 2004; Yoo and Shin 2012). It has been suggested that human IBD may be caused by an autoimmune dysregulation and imbalance of T cells or host responsiveness against a microbial trigger (Mizoguchi, Mizoguchi and Bhan 2003; Singh, et al. 2007). In addition, many studies report that MAP or MAP-specific serum antibodies are detected in the intestine, blood and breast milk of patients...
with CD (Naser et al. 2000; Naser, Schwartz and Shafran 2000; Schwartz et al. 2000; Naser et al. 2004; Sechi et al. 2005). MAP DNA has been detected in retail pasteurized milk samples, even commercially pasteurized milk in the UK. As transmission could occur through milk and other dairy products, further research into the mechanisms underlying MAP pathogenesis in humans is needed (Millar et al. 1996; Ellingson et al. 2005). Despite its possible role in the etiology of CD, there have been few studies examining the interactions between MAP and human cells. Different gene expression patterns or cytokine responses [tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-10] were observed in human monocyte-derived macrophage THP-1 cells by different MAP strains (Motiwala et al. 2006; Borrmann et al. 2011).

In the current study, we applied a microarray-based approach to discover potential immune response markers in a model of murine MAP infection and naturally MAP-infected cattle followed by validation of the genes in human cells. In our previous study, the host responses to MAP infection in mice and cattle were characterized by transcriptional analysis. Significantly regulated genes were filtered and selected via Ingenuity Pathways Analysis (IPA) and evaluated in vitro using MAP-infected THP-1 cells.

MATERIALS AND METHODS

Selection of potential host response genes using transcriptional profiles in MAP-infected mice and naturally MAP-infected cattle

We previously submitted a data set of MAP infection to the Gene Expression Omnibus (GEO) database (GSE62836) for murine study (submitted to PLoS One, Shin et al. 2015a) and to the GEO database (GSE62835) for bovine study (Shin et al. 2015b). For murine study, in brief, 5-week-old C57BL/6 female mice were inoculated intraperitoneally with 300 μl of phosphate-buffered saline (PBS; control group) or a suspension of MAP ATCC 19698 (3 × 10⁷ cells/mouse, MAP-infected group). Spleen samples were taken from five mice in each group, sacrificed at 3 and 6 weeks post-infection (p.i.). Microarray analysis was performed using total RNA from harvested spleen cells and the data were used for identification of differentially expressed genes in MAP-infected mice compared with control mice. Differentially expressed genes were filtered for a log2-fold change >2 with P < 0.05 by biomarker filter and then analyzed by biomarker discovery both of IPA (Ingenuity Systems Inc., Redwood, CA, USA) and GSEA. The genes were further clarified in the IPA biomarker filter module based on the following criteria: fluid – ((all)), disease – ((Infectious Disease, Inflammatory response, Immunological disease)) and species – ((mouse)). Genes selected based on the above criteria were compared with bovine transcriptional profiles and verified in THP-1 cells following infection with MAP using quantitative real-time reverse transcription–PCR (RT-PCR).

Bacterial strain, cell line culture, and infection

MAP ATCC 19698 was cultured as previously described (Cha et al. 2013). For infection of cells, MAP ATCC 19698 was suspended in PBS and used at a dilution of 1 × 10⁷ cells/ml. THP-1 human leukemic monocyte cell line was obtained from KCLB (Korea Cell Line Bank, Seoul, Korea). THP-1 cells were cultured in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and Antibiotic–Antimycotic (all from Gibco Invitrogen, Karlsruhe, Germany) agent at 37°C in humidified air with 5% CO₂. THP-1 cells were differentiated into macrophages by stimulation with phorbol-12-myristate-13-acetate (PMA; Sigma Co., St. Louis, MO, USA) (50 ng/ml) for 72 h, washed with FBS-free RPMI 1640 medium and incubated with 5% FBS-RPMI 1640 medium without antibiotics for 24 h before the experiments. Differentiated THP-1 macrophage cultures (1 × 10⁶ cells/ml) were inoculated with MAP 19698 at a multiplicity of infection (MOI) of 10:1 and incubated for 0, 6, 24 and 48 h. Total RNA was extracted from cells collected at 0, 6, 24 and 48 h p.i..

Quantitative real-time PCR

The total RNA was isolated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. A 2 μg aliquot of total RNA from each sample was reverse-transcribed into cDNA using a Quantitect® Reverse Transcription kit (Qiagen). Quantitative real-time RT-PCRs were performed with 2 μl of cDNA using the Rotor-Gene SYBR Green PCR kit (Qiagen) and Rotor-Gene Q real-time PCR cycler (Qiagen). Cycling parameters were as follows: 95°C for 5 min for one cycle, 95°C for 20 s, and then 60°C for 45 s for 40 cycles. Primers used in real-time PCR are shown in Supplementary Table 1. The gene expression level was normalized by the 2−ΔΔCt method using β-actin as an internal control gene. The relative expression level was compared with the zero-time control to determine the changes in the fold expression of each gene.

Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM), and statistical significance was analyzed by Student’s t-test using the IBM Statistical Package for Social Sciences software (SPSS, version 21, SPSS Inc., Chicago, IL, USA). Differences were considered significant if a value of P ≤ 0.05 was obtained.

RESULTS

Host gene expression in MAP-infected mice and naturally MAP-infected cattle

In our previous study, C57BL/6 mice infected with MAP showed the most severe immunopathological changes at 3 and 6 weeks p.i. by transcriptional profiling and histopathological analysis. As the first step of the data mining process, the ‘up-regulated’ genes were selected using the cut-off value of log2-fold change >2 and P < 0.05. In the second, the genes were imported to the IPA biomarker filter and then analyzed by biomarker discovery both of IPA (Ingenuity Systems Inc., Redwood, CA, USA) and GSEA. The genes were further clarified in the IPA biomarker filter module based on the following criteria: fluid – ((all)), disease – ((Infectious Disease, Inflammatory response, Immunological disease)) and species – ((mouse)). Genes selected based on the above criteria were compared with bovine transcriptional profiles and verified in THP-1 cells following infection with MAP using quantitative real-time reverse transcription–PCR (RT-PCR).

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Table 1. Expression levels of selected genes in MAP-infected mice and naturally MAP-infected cattle.

<table>
<thead>
<tr>
<th>Group</th>
<th>Gene symbol</th>
<th>Gene Name</th>
<th>Location</th>
<th>Family</th>
<th>3 weeks p.i.</th>
<th>6 weeks p.i.</th>
<th>Test1</th>
<th>Test2</th>
<th>Test3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CD14</td>
<td>CD14 molecule</td>
<td>Plasma membrane</td>
<td>Transporter</td>
<td>5.04</td>
<td>2.05</td>
<td>–</td>
<td>2.04</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>CD68</td>
<td>CD68 molecule</td>
<td>Plasma membrane</td>
<td>Other</td>
<td>2.3</td>
<td>2.61</td>
<td>–</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S100A8</td>
<td>S100 calcium-binding protein A8</td>
<td>Cytoplasm</td>
<td>Other</td>
<td>64.76</td>
<td>5.6</td>
<td>–</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>S100A9</td>
<td>S100 calcium binding protein A9</td>
<td>Cytoplasm</td>
<td>Other</td>
<td>46.77</td>
<td>4.65</td>
<td>–</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>ELANE</td>
<td>Elastase, neutrophil expressed</td>
<td>Extracellular space</td>
<td>Peptidase</td>
<td>12.99</td>
<td>5.61</td>
<td>–</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>HMOX1</td>
<td>Heme oxygenase (decycling 1)</td>
<td>Cytoplasm</td>
<td>Enzyme</td>
<td>6.9</td>
<td>2.85</td>
<td>–</td>
<td>2.3</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>LTF</td>
<td>Lactotransferrin</td>
<td>Extracellular space</td>
<td>Peptidase</td>
<td>64.27</td>
<td>7.68</td>
<td>–</td>
<td>3.8</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>TCF7L2</td>
<td>Transcription factor 7-like 2</td>
<td>Nucleus</td>
<td>Transcription regulator</td>
<td>3.29</td>
<td>2.12</td>
<td>–</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

B
|       | CCL4        | Chemokine (C-C motif) ligand 4 | Extracellular space | Cytokine | 2.03 | 2.6 | – | – | – |
|       | CCL5        | Chemokine (C-C motif) ligand 5 | Extracellular space | Cytokine | 2.84 | 3.02 | – | – | – |
|       | HP          | Haptoglobin                  | Extracellular space | Peptidase | 23.85 | 11.88 | – | – | – |
|       | MPO         | Myeloperoxidase              | Cytoplasm | Enzyme | 39.33 | 7.85 | – | – | – |
|       | CXCL9       | Chemokine (C-X-C motif) ligand 9 | Extracellular space | Cytokine | 4.65 | 8.4 | – | – | – |
|       | CXCL10      | Chemokine (C-X-C motif) ligand 10 | Extracellular space | Cytokine | 4.94 | 4.58 | – | – | – |
|       | IFNG        | Interferon, gamma            | Extracellular space | Cytokine | 2.62 | 3.22 | – | – | – |
|       | TFRC        | Transferrin receptor (p90, CD71) | Plasma membrane | transporter | 11.13 | 9.12 | – | – | – |
|       | GPNMB       | Glycoprotein (transmembrane)nmb | Nucleus | Other | 5.49 | 14.22 | – | – | – |
|       | GADD45      | Growth arrest and DNA damage-inducible protein | Nucleus | Enzyme | 12 | 7.36 | – | – | – |
|       | CHI3L1      | Chitinase 3-like 1 (cartilage glycoprotein-39) | Extracellular space | Enzyme | 2.47 | 2.35 | – | – | – |
|       | IGF1        | Insulin-like growth factor1 (somatomedin C) | Extracellular space | Growth factor | 2.14 | 2.21 | – | – | – |
|       | NQO1        | NAD(P)H dehydrogenase, quione1 | Cytoplasm | Enzyme | 2.14 | 2.21 | – | – | – |

Validation of biomarker candidates in THP-1 by quantitative PCR

The 21 selected candidates were evaluated in human THP-1 cells at various time points following MAP infection. No significant change in expression was observed in seven of these genes (CD68, GADD45, GPNMB, HMOX1, IFNG, NQO1 and TCF7L2) at any time point relative to the uninfected control. The expression of CCL4 (3.5-fold up-regulation), CCL5 (2.4-fold up-regulation), CXCL9 (3.5-fold up-regulation) and TFRC (2.1-fold up-regulation) genes was increased at 6 h p.i. but not at later time points, and the expression of CD14 (4.1- and 4.8-fold up-regulation at 24 and 48 h p.i.), CXCL10 (1.8- and 3.4-fold up-regulation at 24 and 48 h p.i.), LTF (1.7- and 1.4-fold up-regulation at 24 and 48 h p.i.), S100A8 (6.2- and 6.7-fold up-regulation at 24 and 48 h p.i.) and S100A9 (2.7- and 3.9-fold up-regulation at 24 and 48 h p.i.) genes was increased at 24 and 48 h p.i. (Fig. 1). CHI3L1 (2.6-fold up-regulation) and HP (4.3-fold up-regulation) genes showed increased expression only at 48 h after MAP infection (Fig. 1), while ELANE (1.7- and 1.8-fold down-regulation at 24 and 48 h p.i.), IGF1 (2.3- and 1.5-fold down-regulation at 24 and 48 h p.i.) and MPO (2.1- and 1.8-fold down-regulation at 6 and 48 h p.i.) genes showed down-regulation. Therefore, among the selected genes from the transcriptomes of MAP-infected mice, the expression of 14 of these genes was also significantly up- or down-regulated in human THP-1 cells following MAP infection.

DISCUSSION

Although the oral route is the natural infection route of MAP in cattle, intraperitoneal injection has been frequently utilized in MAP infection of mice. and our previous murine study also challenged MAP intraperitoneally, because earlier studies demonstrated that low infectivity rates are the main problem with the oral administration of MAP in the mouse model (Mutwiri et al. 1992; Veazey et al. 1995). In the previous study, we analyzed transcriptional profiles induced in the murine spleen, wherein the innate and adaptive immune systems combined, following MAP infection. The expression patterns of 21 candidate genes were analyzed at various time points p.i. CD68 and CD14, both macrophage markers, showed different expression patterns. CD68 was not significantly altered by MAP infection in THP-1 cells. However, in these experiments, PMA-stimulated THP-1 macrophage cells were used and CD68 was already expressed in these cells prior to infection, which may explain why no further change was observed. The expression of CD14...
Figure 1. Gene expression changes between MAP-infected THP-1 cells and uninfected control. The selected genes were put into three groups as follows: A, genes up-regulated in both mice and cattle; B, genes up- and down-regulated in mice and cattle, respectively; and C, up-regulated and no significant changes in mice and cattle, respectively. The relative expression level was normalized with the \( \beta \)-actin expression level relative to the zero time control by the \( 2^{-\Delta\Delta CT} \) method. Significance level set at \( P \leq 0.05 \) (\( * P \leq 0.05; ** P \leq 0.01; *** P \leq 0.001 \)).
increased at 24 and 48 h p.i. A role for CD14 has been reported in the regulation of monocyte/macrophage apoptosis so up-regulation of CD14 may have an effect on levels of apoptosis during infection (McClure et al. 1987).

Increased expression of S100A8 and S100A9 [also termed myeloid-related protein (MRP) 8 and MRP14] was observed, consistent with data demonstrating release of these proteins by activated phagocytes during mycobacterial infection both in vitro and in vivo (Pechkovsky et al. 2000; Frosch et al. 2004). Cows with MAP-specific antibodies showed up-regulation of MRP8 and MRP14 (Shin, et al. 2015b). They form stable complexes (MRP8/14) and are found in a variety of inflammatory conditions, including rheumatoid arthritis, allograft rejections and inflammatory bowel and lung diseases (Kerkhoff, Klempt and Sorg 1998). Therefore, their potential role in inflammatory disorders including CD has already been highlighted (Lugering et al. 1995; Foell et al. 2004). The LTF (lactoferrin) gene was also slightly up-regulated in THP-1 cells infected with MAP. Serum LTF was strongly and inversely associated with an increasing bacterial index of M. leprae in leprosy patients (Muruganand et al. 2004). Of group A, the TCF7L2 gene was down-regulated at 6 h p.i., but there were no statistically significant responses. TCF7L2 was suggested to modulate hepatic glucose metabolism in mammals, thus contributing to the development of type 2 diabetes (Oh et al. 2012).

Although expression of Th1-type chemokines and interferon-γ (IFN-γ) was not observed in naturally MAP-infected cows, with the exception of down-regulation of CCL4 and CCL5 in Test1 of cows, Th1-type chemokines were increased at 6 h p.i. in THP-1 cells infected with MAP; in particular, expression of CXCL10 was gradually increased over the time course. Th1-type chemokines and chemokine receptors (CCL5 and CXCR3) show increased infiltration in granulomas of CD patients (Oki et al. 2005), and a role for CCL5 and related chemokine receptors was suggested in the pathogenesis of JD in THP-1 cells stimulated with MAP isolates (Motiwala et al. 2006). Our results which indicate up-regulation of Th1-type chemokines (CCL4, CCL5, CXCL9 and CXCL10) in MAP-infected THP-1 cells are consistent with previous data and suggest a relationship between Th1-type chemokines and their receptors and the host response to MAP infection. IFN-inducible protein 10 (IP-10, CXCL10) has been reported to be up-regulated in some Th1-mediated inflammatory disorders in humans, including type 1 diabetes mellitus (Cossu et al. 2013), rheumatoid arthritis (Patel, Zachariah and Whichard et al. 2001), multiple sclerosis (Balashov et al. 1999), sarcoid granulomatous disease (Beard et al. 1999) and IBD (Zwick et al. 2002). CXCL10 has been described as an alternative or adjunct biomarker to IFN-γ, showing high expression in serum of tuberculosis- and tuberculosis–HIV-co-infected patients (Jordao et al. 2008). Detection of CXCL9 and CXCL10 by quantitative PCR was also reported as a potential method to enhance diagnostic sensitivity in human tuberculosis (Flynn and Chan 2003; Magee et al. 2012). Monokine induced by IFN-γ (MIG, CXCL9) was also induced in a murine model of IBD (McKinney et al. 2000; Ito et al. 2006). This suggests that Th1-type chemokines may be valuable biomarkers for MAP infection.

Haptoglobin (HP), an acute-phase protein, has been demonstrated to chemoattract monocytes, to modulate the immune response and to have anti-inflammatory activities (Beard et al. 2000; Madsen, Graversen and Moestrup 2001; Moura et al. 2012). HP was suggested as an obesity and inflammatory marker in its role as a novel monocyte chemoattractant (Beard, et al. 2000). Furthermore, serum HP was used within a disease activity index in a model of murine IBD (Ito et al. 2006) and proposed as an independent prognostic factor in epithelial ovarian cancer patients (Hines et al. 2014). Elevated serum levels of Chitinase 3-Like-1 (CHI3L1) have been correlated with increased disease severity and a poorer prognosis for many diseases, including cancers, autoimmune diseases and chronic inflammatory conditions (Ravva and Stanker 2005). Our findings of up-regulation of HP and CHI3L1 at 48 h p.i. in MAP-infected THP-1 also suggest that these genes could provide a useful measure in progression of MAP infection. Expression of the TFRC gene was increased at 6 h p.i. and maintained at 24 and 48 h p.i. TFRC is known as an early phagosome marker, and its expression may be affected by the interference of MAP in the phagosome maturation process (Ruhl and Collins 1995). TFRC was also up-regulated at 6 h p.i. in Raw 264.7 cells infected with MAP (Cha et al. 2013). In addition, fecal shedding of naturally infected cows showed down-regulation of the MPO (myeloperoxidase) gene (Test1 and Test3, Table 1), and the gene was also down-regulated in THP-1 infected with MAP. The MPO gene was known to generate reactive oxidants and that has been implicated in mycobacterial killing (Borelli et al. 1999). Down-regulation of the MPO gene could be suspected to be associated with resistance of MAP against the host defense response. In the present study, THP-1 cells were treated with PMA for differentiation before infection with MAP. Even though the phenotype of THP-1 differentiated using a modified protocol (i.e. incubated for a further 5 days in fresh media after treatment with PMA, but a further 1 day incubation in our case) shared characteristics of primary monocyte-derived macrophages (Daigneault et al. 2010), the responses of natural MAP infection in human macrophages could be different from those in THP-1.

In the present study, we can see the potential host responses including up-regulation of genes related to immune response (CD14, S100A8, S100A9, LTF, HP and CHCIL3), up-regulation of Th1-polarizing factor (CCL4, CCL5, CXCL9 and CXCL10), down-regulation of genes related to metabolism (ELANE, IGF1, TCF7L2 and MPO) and no significant response of other genes (GADD45a, GPNMB, HMOX1, IFNG and NQO1) in human macrophage cells infected with MAP, which were selected by an in silico genomic technique (Biomarker Discovery of IPA) in MAP-infected mice.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSPD online.

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**Conflict of interest.** None declared.

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