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Mycobacterium avium Subspecies paratuberculosis Recombinant Proteins Modulate Antimycobacterial Functions of Bovine Macrophages

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Abstract

It has been shown that Mycobacterium avium subspecies paratuberculosis (M. paratuberculosis) activates the Mitogen Activated Protein Kinase (MAPK) p38 pathway, yet it is unclear which components of M. paratuberculosis are involved in the process. Therefore, a set of 42 M. paratuberculosis recombinant proteins expressed from coding sequences annotated as lipoproteins were screened for their ability to induce IL-10 expression, an indicator of MAPKp38 activation, in bovine monocyte-derived macrophages. A recombinant lipoprotein, designated as MAP3837c, was among a group of 6 proteins that strongly induced IL-10 gene transcription in bovine macrophages, averaging a 3.1-fold increase compared to non-stimulated macrophages. However, a parallel increase in expression of IL-12 and TNF-α was only observed in macrophages exposed to a subset of these 6 proteins. Selected recombinant proteins were further analyzed for their ability to enhance survival of M. avium within bovine macrophages as measured by recovered viable bacteria and nitrite production. All 6 IL-10 inducing MAP recombinant proteins along with M. paratuberculosis cells significantly enhanced phosphorylation of MAPK-p38 in bovine macrophages. Although these proteins are likely not post translationally lipidated in E. coli and thus is a limitation in this study, these results form the foundation of how the protein component of the lipoprotein interacts with the immune system. Collectively, these data reveal M. paratuberculosis proteins that might play a role in MAPK-p38 pathway activation and hence in survival of this organism within bovine macrophages.

Introduction

Mycobacterium avium subsp. paratuberculosis (M. paratuberculosis) is a pathogen with a broad host-range characterized by the capacity to evade the immune system and to cause severe chronic intestinal granulomatous inflammation. Although it can infect multiple species, it is...
primarily a disease problem in ruminants, which include cattle, sheep and goats [1]. The lack of a complete understanding of the host immune response against this pathogen has hindered the development of an effective vaccine. A sustained research effort has been focused on the biology of *M. paratuberculosis* to improve our knowledge and understanding of the infection process [1, 2]. The dairy industry incurs substantial economic losses due to reduced milk production, premature culling and reduced slaughter value [3]. The bacterium is shed in the feces and milk of infected animals, primarily in the clinical phase of disease [4]. Transmission of disease can occur by ingestion of the bacterium through manure-contaminated feedstuffs and pastures or by colostrum and milk, passed from the infected dam to the calf [4, 5].

Pathogenic mycobacteria interfere with the phagosome maturation process, but the precise mechanism has not been fully detailed [6]. *M. paratuberculosis* is known to survive within macrophages by impairing nitric oxide production [7] and has been shown to resist physiological concentrations of nitric oxide [8]. However, *M. paratuberculosis* appears susceptible to IFN-γ induced activation of cultured macrophages which supports the fact that elevated levels of IFN-γ are present in cows with subclinical Johne’s disease and the cytokine appears important for controlling mycobacterial infection [9]. Nonetheless, addition of IFN-γ to *M. paratuberculosis*-infected macrophage cultures does not appear to promote killing of the bacteria [7, 8, 10]. Another mechanism by which *M. paratuberculosis* survives within macrophages is by inhibiting phagosome acidification and maturation. Phagosomes containing *M. paratuberculosis* do not accumulate lysosomal markers and do not acidify lower than pH 6.3 in J774 macrophages [11], indicating a failure of the phagosome to mature into a phagolysosome.

Previous studies showed that *M. paratuberculosis* infection of bovine macrophages results in increased IL-10 transcription and decreased IL-12 transcription [12, 13], a gene expression pattern that promotes interaction with the innate immune receptor Toll-like receptor 2 (TLR2) and activation of intracellular immune cytokine regulator, MAPKp38, in bovine macrophages [14, 15]. Mitogen Activated Protein kinases (MAPK) are stress activated kinases with the MAPKp38 kinase existing as four isoforms, alpha, beta, gamma and delta [16]. In the case of p38 alpha, it is the sites Thr180/Tyr182 that become dual phosphorylated, signaling activation within the cell. MAPKp38 is activated as a result of cellular stresses, most notably the presence of inflammatory cytokines [16]. Because the MAPKp38 pathway is a mechanism for suppression of antimicrobial responses within macrophages, activation of this pathway could enable intracellular survival of *M. paratuberculosis*. It also induces production of the anti-inflammatory cytokine interleukin (IL)-10 [13, 14]. Induction of IL-10 has been described as playing key roles in dampening the immune system, favoring *M. paratuberculosis* survival within host cells [17].

Conversely, tumor necrosis factor (TNF)-α is produced by macrophages and dendritic cells as a primary response to infections and tissue damage [18]. TNF-α plays an important role in activation and recruitment of leukocytes to inflamed tissue [18], and has been demonstrated to be involved in the host-defense against *M. tuberculosis* [19]. However, TNF-α is also associated with excessive inflammation and immunopathology in infections and autoimmune diseases. The specific role of the MAPKp38 pathway in phagosome maturation during mycobacterial infection is not completely understood; however, it has been shown that *M. paratuberculosis* infection of cultured bovine macrophages results in a rapid phosphorylation of MAPKp38 [14]. The pathogenic pathway that initiates with *M. paratuberculosis*-TLR2 engagement leading to activation of the MAPKp38 pathway and culminating with high levels of IL-10 production can also be exploited to rationally design a critically needed vaccine against *M. paratuberculosis*.

Lipoproteins are involved in a variety of functions including cell wall synthesis, adhesion, transmembrane signaling and anchoring proteins to the cell membrane [20]. It is known that mycobacterial lipoproteins are recognized by TLR [21]. Mannosylated lipoarabinomannan
Man-LAM is a mannose-capped lipoglycan cell wall component of pathogenic mycobacteria that was previously shown to induce strong expression of IL-10 [22]. Since Man-LAM contains lipid and lipoproteins are part of a larger group of microbial molecules that are called pathogen-associated molecular patterns (PAMPs) that interact with TLRs [23–25], we reasoned that lipoproteins may be involved in MAPKp38 activation. Furthermore, two lipoproteins that result in attenuation of M. tuberculosis when disrupted include LpqS [26] and LspA [25]. Therefore, the M. paratuberculosis genome was searched for all genes annotated as lipoproteins and they were expressed and purified from E. coli and used to test activation of MAPKp38, ability to stimulate expression of IL-10 and its capacity to prevent killing of M. avium subspecies avium (M. avium) by bovine macrophages. At least two recombinant proteins were discovered that have modular effects on macrophage-mycobacterial interactions. Genes encoding these recombinant proteins are considered targets for constructing directed knockout mutations to test attenuation in bovine macrophages.

Materials and Methods

Monocyte isolation and macrophage generation

All work involving animals was conducted in accordance with the recommendations in the institutional guidelines and approved animal care and use committee (IACUC) protocols at Washington State University. All other experiments were carried out in accordance with the Washington State Universities' Institutional Biosafety Committee (IBC) approved protocol number 1190 along with the National Animal Disease Center's IBC-0261 protocol. Blood samples used for isolation of monocytes were collected from three healthy adult Holstein dairy cows that tested negative for paratuberculosis as determined by culture and IS900 PCR analysis of fecal samples. Peripheral blood mononuclear cells were isolated by centrifugation on a Percoll density gradient as described [12]. Briefly, blood was layered onto 50 mL conical tubes containing Histopaque 1077 (Sigma-Aldrich, USA), and following density gradient centrifugation (500 x g for 20 minutes) at room temperature, peripheral blood mononuclear cells (PBMC) were collected. Thereafter, PBMCs were washed twice with sterile phosphate-buffered saline (PBS; Invitrogen, Life Technologies, USA) before resuspending cells in PBS. Monocytes were then isolated using microbeads conjugated with mouse anti-human CD14 antibody (isotype mouse IgG2a; Miltenyi Biotec Ltd., San Diego, USA), which has been shown to be cross-reactive with bovine monocytes [27]. The isolation was performed according to the manufacturers' instructions. The identity and purity of monocytes (>97%) was determined by flow cytometry using an anti-CD14 fluorescein-labeled antibody (data not shown). Purified monocytes were seeded at 2x10^6 per well in 12-well tissue culture plates containing Dulbecco's Modified Eagle medium with high glucose (Invitrogen, Life Technologies, USA) with 10% heat inactivated fetal calf serum (Sigma-Aldrich, USA), gentamicin (5 mg/ml; Sigma-Aldrich, USA), 100 ng/mL GM-CSF (Kingfisher, USA) and 1mM β-mercaptoethanol (Sigma-Aldrich, USA). Subsequently, cells were incubated at 37°C in a 5% CO₂ humidified atmosphere. On day 7 confluent macrophages were used in all described experiments.

Culture conditions for Mycobacterium avium subspecies

M. paratuberculosis K-10 and M. avium subspecies avium strain ATCC 35716, originally isolated from cattle, was obtained from the American Type Culture Collection, USA. M. avium cells were grown to a concentration of approximately 10^7 CFU/ml, washed, and resuspended in Middlebrook broth containing Oleic Albumin Dextrose Catalase Growth Supplement, and Tween 80. Viability of the organisms added to macrophage cultures varied between 85% and 95% as determined by propidium iodide exclusion (data not shown). Immediately before
addition to macrophage cultures, organisms were washed in warm PBS and resuspended in RPMI1640 medium without antibiotics. *M. paratuberculosis* was cultured only for the MAPK-p38 activation experiment.

**Production and purification of recombinant *M. paratuberculosis* proteins**

*M. paratuberculosis* genes annotated as lipoproteins were selected for cloning into the pMAL-c2x expression vector and transformed into *E. coli* DH5α. All clones were confirmed to be correct and in-frame with the maltose binding protein by DNA sequencing. To obtain the MBP-lacZ alpha peptide control protein, the native pMAL-c2x vector, without a cloned insert, was expressed in the same way as the *M. paratuberculosis* recombinant clones. Confirmed transformants were cultured, induced with IPTG and recombinant fusion proteins purified as described previously [28]. The only modification was that proteins eluted off the amylose resin column were collected and loaded onto a second amylose column to maximize removal of potential LPS contamination. LPS contamination was ruled out for a selection of these proteins using the Limulus amebocyte lysate gel clot assay (Lonza).

**Infection and RNA extraction of cultured macrophages**

*M. avium* organisms (MOI: 10 bacilli/macrophage) were added to cell cultures with and without addition of *M. paratuberculosis* recombinant proteins (5 μg/ml) and incubation was continued at 37°C in 5% CO₂. Cellular mRNA was harvested from plates at 2 hours using the RNeasy kit (Qiagen, USA) following the manufacturer instructions. The RNA purity was assessed by measuring the 260/280 ratio with Nanodrop (Nanodrop Products, Wilmington, USA). Integrity of RNA preparations was assessed by use of RNA agarose gel electrophoresis. As a control for DNA contamination, a direct PCR was performed to confirm the absence of β-actin amplification in RNA samples. RNA samples were stored in 10 μL aliquots at -80°C until further processing.

**Determination of cytokine gene expression by quantitative real-time PCR**

Genomic DNA was removed from mRNA samples by use of a commercial kit (RNeasy plus Mini Kit, Qiagen, USA) following the manufacturer instructions immediately after mRNA isolation from cultured macrophages. First-strand cDNA was synthesized by use of a commercial kit (Script cDNA Synthesis Kit, Bio-Rad, USA) following the manufacturer instructions. Then, cDNA was diluted to 100 μl total volume and SYBR green master mix was added (Power SYBR Master Mix, Life Technologies, USA). Samples were analyzed in triplicate in a 96-well optical reaction plate. Each sample contained 5 μl of cDNA diluted to 1:10 in DNAse free water and 15 μl of SYBR green master mix. Primers (Table 1) were designed using a web-based program; [http://biotools.umassmed.edu/bioapps/primer3_www.cgi](http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Gene expression was evaluated as relative fold expression using the ΔΔCt method. GAPDH was used as an endogenous control to normalize the gene expression input. Preliminary results showed no variation in the expression of GAPDH in macrophages treated with the chemical MAPKp38 inhibitor (SB203580, Sigma-Aldrich, USA), or DMSO to untreated macrophages (data not shown).

**Determination of nitric oxide production**

After 24-hour incubation of *M. paratuberculosis*-derived peptides (5 μg/mL) with primary bovine macrophages, nitrite (i.e., the stable by-product of nitric oxide generated by phagocytes) was measured in culture supernatants. Fifty microliters of supernatant was mixed with 200 μL.
of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% H₃PO₄) and incubated at 25°C for 10 minutes. Absorbance was determined at 540 nm, and absorbance readings were converted to micromolar concentrations by comparing results for samples with results for a standard curve generated by use of concentrations ranging from 1.5 to 200 μM of NaNO₂.

Phagocytosis and intracellular survival of mycobacterial organisms

*M. avium* organisms were used instead of *M. paratuberculosis* in the survival assay because macrophages have been shown to more effectively kill *M. avium* organisms therefore increasing the assay sensitivity. Monocyte-derived macrophages attached to coverslips were stained with Ziehl-Neelsen carbolfuchsin stain (Sigma, St Louis, MO) for presence of mycobacteria and other acid-fast organisms. The percentage of macrophages 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 (BackLight kit, Invitrogen, Carlsbad, CA). This test has previously been reported to provide a rapid and reliable method for differentiating live vs. dead *M. avium* organisms [14]. Macrophages were preincubated with or without *M. paratuberculosis*-derived recombinant proteins (5 μg/mL) for 2 hours and then infected with *M. avium*. After 72 hours, macrophages were washed twice in PBS solution and then 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, coverslipped, and examined on a fluorescent microscope (40X objective) by using 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 per treatment group.

### Determination of MAPKp38 phosphorylation by enzyme-linked immunosorbent assay (ELISA)

An ELISA kit (InstantOne, eBioscience, USA) was used to measure phosphorylated levels of MAPKp38α in bovine macrophage lysates post treatment with either ovalbumin (OVA, Life Technology, USA) at 5 μg/mL, live *M. paratuberculosis* organisms at 10:1 MOI or *M. paratuberculosis* recombinant proteins at 5 μg/mL. Resting macrophages were used as the background control. A modified protocol was used such that 300 μL cell lysates were added to completely cover a single well in a standard 12-well plate. Equal parts cell lysate plus capture and detection antibody reagents were added simultaneously to the InstantOne assay plate. After 1 hour of incubation at 37°C, the wells were washed and detection solution (supplied by the kit) was applied for 20 minutes in the dark. Absorbance was measured at 450 nm in a standard ELISA plate reader. Positive control cell lysate and negative control (cell lysis buffer), as well as untreated lysates, confirmed antibody efficacy. Experimental replicates were done in triplicate.

### Table 1. Primers used for qRT-PCR.

| Gene       | Forward 5’- Primer | Reverse 5’- Primer | Accession no. |
|------------|--------------------|--------------------|---------------|
| TNF-α      | TCAACACTCAGGCCTTTGCTCA-3’ | GTCGGCTACAACGTGGGTACC-3’ | AC000180     |
| IL-10      | CGGCTGGCCGCCTCTTCA-3’ | TCACCTTCTCCACGCGGCTCT-3’ | P43480       |
| IL-12 (p40) | TCGGCCATTGAGGCTCA-3’ | ACACAACCTGAGGGAAGTAG-3’ | P46282       |
| GAPDH      | GAAACCTGCAATATGAGATGAGAC-3’ | TGTAGCCTAGATAAGCCCTTGAGAG-3’ | P10096       |

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Statistical analysis

All tests were performed in triplicate and results of at least three separate experiments were evaluated. Mann-Whitney statistical test was used to verify normal distribution of the data. Results were expressed as mean ± SD. Differences between cell cultures incubated with *M. paratuberculosis* recombinant proteins with or without adding *M. avium* were analyzed by use of the paired student t-test. *P* < 0.05 was considered to be statistically significant.

Results

*M. paratuberculosis* recombinant proteins stimulate cytokine transcription

Since it is known that the MAPKp38 pathway induces expression of IL-10, while at the same time suppressing IL-12 expression, we tested transcription of these cytokines in macrophages after exposure to *M. paratuberculosis* recombinant proteins. It is further known that lipid containing molecules such as the 19-kDa antigen and trehalose 6, 6’-dimycoclate activate the TLR2 pathway [24, 29], thus we reasoned that *M. paratuberculosis* lipoproteins may also be involved in TLR2 interactions. Therefore the genome sequence of *M. paratuberculosis* [30] was searched for genes annotated as lipoproteins. A total of 51 genes satisfied these criteria. Of these, 42 genes (82%) were successfully cloned and expressed in *E. coli* (Table 2). Macrophages were incubated with or without these purified fusion proteins and analyzed for cytokine expression including TNF-α, IL-12 and IL-10 transcription (Table 2). The results show 6 of 42 proteins had greater than 2 fold increase of IL-10 transcription over background. These six include MAP0261c, MAP0584, MAP2322c, MAP3615c, MAP0981c and MAP3837c (Fig 1A).

MAP2322c also stimulated transcription of both IL-12 and TNF-α above the level of the control, whereas MAP0981c stimulated transcription of only IL-12 (Fig 1B and 1C). These data initially suggest MAP0261c, MAP0584 and MAP3837c are the primary candidates for stimulating MAPKp38 phosphorylation.

MAP1761c increases survival of *Mycobacterium avium* in macrophages

Although it has been shown that some *M. avium* strains can survive within human and murine macrophages [31], other studies have shown that *M. avium* is more susceptible to killing within cultured bovine macrophages than is *M. paratuberculosis* [10, 32]. Therefore, to evaluate the effect of *M. paratuberculosis* proteins on *M. avium* survival within bovine macrophages, cells were preincubated in the presence or absence of recombinant proteins for 2 hours and then incubated with *M. avium* for 72 hours, which is the time needed to kill approximately half of the *M. avium* inoculum [10]. When macrophages were exposed to MAP1761c, *M. avium* survival was enhanced with 71±4% of the cells surviving after 72 hours (Fig 2A). MAP3837c seemed to also have a preservation effect as 60±10% of the inoculum survived in macrophages exposed to that protein. In contrast, only 32±5% *M. avium* cells survived after preincubation with MAP0261c (Fig 2A).

MAP2322c increases production of nitric oxide

Nitric oxide (NO) is a reactive signaling molecule and an important inflammatory mediator, which acts as a cytotoxic agent in addition to modulate immune responses and inflammation through multiple immune networks. Our results showed that compared to macrophages incubated with *M. paratuberculosis* and the control peptide LacZ, only MAP2322c shows significant increase in production of NO (Fig 2B).
Table 2. *Mycobacterium avium* subspecies *paratuberculosis* protein effect on NO2 and cytokines.

| Protein    | Description                          | Conc. NO2 (A550) (ug/cc) | NO2 fold change | TNF-α SD | IL-12 fold change | IL-10 fold change |
|------------|--------------------------------------|---------------------------|-----------------|----------|-------------------|-------------------|
| MAP0261c   | 19-kDa lipoprotein, LpqH             | 0.048                     | 2.7             | 1.527    | 0.498             | 1.318             |
| MAP0584    | Lipoprotein, LprH                     | 0.067                     | 3.8             | 2.11     | 0.749             | 1.132             |
| MAP2009    | Zn-dependent hydrolase                | 0.04                      | 2.3             | 3.182    | 1.951             | 1.04              |
| MAP3268    | Small heat shock protein              | 0.036                     | 2.0             | 0.885    | 0.223             | 0.56              |
| MAP1138c   | Lipoprotein, LprG                     | 0.051                     | 2.9             | 1.826    | 0.465             | 2.231             |
| MAP0474c   | Lipoprotein, LpQE                     | 0.09                      | 5.1             | 1.067    | 0.372             | 0.103             |
| MAP2048    | Lipoprotein, LppO                     | 0.083                     | 4.7             | 1.414    | 0.695             | 0.052             |
| MAP2522    | Lipofamily protein, LprE              | 0.065                     | 3.7             | 1.155    | 0.578             | 1.032             |
| MAP3883c   | beta-lactamase protein                | 0.049                     | 2.8             | 3.08     | 1.711             | 2.097             |
| MAP1194c   | 27-kDa lipoprotein                    | 0.07                      | 4.0             | 3.401    | 0.268             | 2.122             |
| MAP3417c   | Lipoprotein, LpqC                     | 0.048                     | 2.7             | 2.655    | 1.573             | 1.117             |
| MAP0466c   | Lipoprotein, LpqF                     | 0.104                     | 5.9             | 1.079    | 0.473             | 0.308             |
| MAP2017    | Lipoprotein, LppN                     | 0.077                     | 4.4             | 0.625    | 0.251             | 0.331             |
| MAP2498c   | Lipoprotein, LprB                     | 0.063                     | 3.6             | 1.185    | 0.636             | 1.041             |
| MAP3688    | beta-glucosidase, LpqI                | 0.078                     | 4.4             | 1.911    | 0.66              | 1.047             |
| MAP0989    | Hypothetical protein, LpqU            | 0.084                     | 4.8             | 1.459    | 1.062             | 1.06              |
| MAP1840    | Lipoprotein, LppK                     | 0.067                     | 3.8             | 7.671    | 2.796             | 3.267             |
| MAP0440c   | Hypothetical protein, LpqG            | 0.09                      | 5.1             | 2.914    | 1.792             | 1.476             |
| MAP1909    | Lipoprotein, LppM                     | 0.075                     | 4.2             | 0.06     | 0.028             | 0.928             |
| MAP2322c   | Hypothetical protein, LppS            | 0.096                     | 5.4             | 5.688    | 3.152             | 8.496             |
| MAP3615c   | Hypothetical protein, LprO            | 0.083                     | 4.7             | 1.274    | 0.79              | 1.09              |
| MAP0981c   | Lipoprotein, LppN family protein      | 0.086                     | 4.9             | 1.698    | 0.823             | 5.149             |
| MAP2216c   | Lipoprotein, LppR                     | 0.047                     | 2.7             | 1.016    | 0.181             | 0.102             |
| MAP3056    | pknH-like protein, LpqA               | 0.065                     | 3.7             | 8.09     | 5.651             | 9.111             |
| MAP1761c   | peptidase M75 protein                 | 0.043                     | 2.4             | 1.761    | 0.602             | 1.29              |
| MAP1781    | Lipoprotein, LppI                     | 0.036                     | 2.0             | 3.984    | 0.483             | 3.593             |
| MAP0670    | D-alanyl-d-alanine dipeptidase, LpqR  | 0.036                     | 2.0             | 0.781    | 0.508             | 0.663             |
| MAP2103c   | LppP/LprE lipoprotein family          | 0.049                     | 2.8             | 3.364    | 1.737             | 2.761             |
| MAP3481    | Histidine phosphatase, LpqD           | 0.037                     | 2.1             | 1.049    | 0.317             | 1.058             |
| MAP3041    | Dehydrogenase, LppZ                   | 0.057                     | 3.2             | 1.47     | 0.364             | 1.436             |
| MAP3908    | Lipoprotein peptidase, LpqM           | 0.044                     | 2.5             | 1.033    | 0.259             | 1.022             |
| MAP1670c   | L, D-transpeptidase, LppS             | 0.062                     | 3.5             | 3.9      | 2.122             | 3.251             |
| MAP3837c   | Hypothetical protein, LpqJ            | 0.062                     | 3.5             | 1.627    | 0.534             | 1.288             |
| MAP1604c   | 19-kDa lipoantigen, LppE              | 0.053                     | 3.0             | 2.893    | 1.02              | 3.826             |
| MAP2497c   | Lipoprotein, LprC                     | 0.065                     | 3.7             | 1.864    | 0.821             | 2.165             |
| MAP2548c   | Solute binding protein, LpqY          | 0.063                     | 3.6             | 2.469    | 1.359             | 3.666             |
| MAP3906    | Amidohydrolase, LpqL                  | 0.049                     | 2.8             | 1.607    | 1.258             | 1.104             |
| MAP2417c   | Hypothetical protein, LppJ            | 0.043                     | 2.4             | 2.773    | 1.078             | 0.738             |
| MAP2539c   | Substrate binding protein, LpqZ       | 0.043                     | 2.4             | 1.592    | 1.239             | 1.163             |
| MAP3907    | Peptidase M28, LpqL                   | 0.064                     | 3.6             | 0.428    | 0.395             | 3.145             |
| MAP1216c   | Lipoprotein, LpqQ                     | 0.069                     | 3.9             | 0.136    | 0.062             | 3.064             |
| MAP1397    | Hypothetical protein, LprJ            | 0.056                     | 3.2             | 0.138    | 0.096             | 1.401             |
| LacZ       | Non-mycobacterial control             | 0.08                      | 4.5             | 1.542    | 0.415             | 0.579             |

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Fig 1. Cytokine expression levels of bovine macrophages in response to MAP recombinant proteins. Shown are IL-10 (A), IL-12 (B) and TNF-α (C), mRNA transcription levels in response to 2-h incubation with MAP Proteins Activates MAPKp38 and Induced IL-10 Expression.
The MAPKp38 pathway is activated by *M. paratuberculosis* proteins

The effects of *M. paratuberculosis* recombinant proteins on phosphorylation of MAPKp38 in bovine macrophages were investigated. Compared to macrophages alone or macrophages incubated with OVA, recombinant proteins as well as live *M. paratuberculosis* cells significantly enhanced phosphorylation of MAPKp38 (Fig 3). However, only MAP0981c stimulated phosphorylation at a higher level than *M. paratuberculosis* cells. These recombinant proteins consistently and reproducibly activated MAPKp38. In contrast, MAPKp38 phosphorylation was attenuated after incubation of macrophages with MAP1761c (data not shown) a protein that failed to induce IL-10 expression (Table 2).

**Discussion**

Previous studies have suggested that Man-LAM purified from *M. paratuberculosis* may interact with mannose receptors on macrophages to promote IL-10 production and inhibit phagosome acidification [22]. This glycolipid complex was not shown to activate the MAPKp38 pathway, but it is known that *M. paratuberculosis* itself activates this pathway [14]. In the present study, we identified specific *M. paratuberculosis* proteins that consistently activate MAPKp38 in a manner similar to *M. paratuberculosis* whole cells. Although Man-LAM demonstrated a much higher fold increase (15-fold) in IL-10 expression [22] than did the proteins tested here, MAP0584 and MAP3837c did show over 3-fold increase in IL-10 expression by macrophages. MAP1761c did not induce expression of IL-10 (Table 2), but appears similar to MAP3837c in that it appears to inhibit killing of *M. avium* within macrophages, although MAP3837c was not statistically significant. Inhibition of killing by these proteins are independent of NO production (Fig 2B). MAP1761c is a predicted periplasmic lipoprotein involved in iron transport and contains the peptidase M75 motif on the C-terminal half. This motif was shown to contain proteolytic activity in *Pseudomonas aeruginosa* although its active site residues have yet to be defined [33]. MAP3837c is annotated as a hypothetical lipoprotein so it is unclear what specific role it may play in the bacterial cell, particularly after engagement with the host macrophage, but its participation in MAPKp38 signaling is apparent.

Our previous data suggest IL-10 is a mediator of *M. paratuberculosis* survival in macrophages and may suggest the TLR2-MAPKp38 signaling pathways are involved in suppression of bacterial killing [15]. We suspect that TLR2 is a key receptor that interacts with *M. paratuberculosis* in some way to prevent it from being killed. Thus, demonstrating which *M. paratuberculosis* protein molecule interacts with TLR2 is a critical first step. Although Man-LAM induces a lengthy IL-10 response [22], preliminary studies suggest it does not interact with TLR2. Unfortunately, demonstration of the lipoproteins and their interaction with TLR2 are more difficult to evaluate. Dose and time are critical for each protein and for this assay lipoproteins are essential. Future studies include testing MAPKp38 phosphorylation after exposure to MAP1761c and MAP3837c. This will include Western blotting with and without the addition of anti-TLR2 antibody for blocking. In addition, an alternative approach that can be pursued to verify the interaction of the proteins studied here with TLR2 is to use the HEK-Blue Detection Kit (Invivogen) that is designed to provide a sensitive and reliable method to screen and validate TLR agonists.
Fig 2. MAP1761c promotes survival of *M. avium* in bovine macrophages. Macrophages were incubated with or without recombinant proteins (5 \(\mu\)g/ml) for 2 h and then infected with *M. avium* for 72 h. (A) Preincubation of macrophages with MAP1761c resulted in 71\(\pm\)4% viability. The other lipoproteins shown...
It should be noted that because the recombinant proteins were expressed in *E. coli*, it is unlikely they were post-translationally lipidated. While all bacteria have the machinery to lipidate proteins, *E. coli* is generally viewed as not suitable for producing lipid-modified mycobacterial proteins. Purification of this extensive set of proteins from the *M. paratuberculosis* host in quantities that could be analyzed was not feasible. Other groups have been successful in producing recombinant forms of the *M. tuberculosis* lipoproteins LppX and LprF using the surrogate host *M. smegmatis* for structural studies [34, 35]. This is the strategy we will pursue with a selection of the best candidates identified in the current study. Nonetheless, with this limitation in mind, we can still make inferences regarding the protein component of the lipoprotein and its immune stimulatory capabilities. However, we acknowledge it is likely the effects observed using these recombinant proteins would either be enhanced or unexpectedly altered if using the native lipidated protein.

Others have shown that IL-12 transcription is increased in *M. paratuberculosis* infected macrophages within 6 hours and this expression remains high through 24 hours but then decreases to background levels by 72 hours post infection [10]. This suggests that *M. paratuberculosis*-infected macrophages rapidly produce IL-12 to enhance the developing T cell response. As shown in Table 2, a set of recombinant proteins consistently increased the transcription of IL-12 and TNF-α but had little effect on IL-10 transcription. Moreover, these proteins failed to activate MAPKp38. It would be reasonable to assume that *M. paratuberculosis* contains a cohort of lipoproteins with pro-immune effects but that this is counterbalanced by the presence of anti-inflammatory and immunomodulatory lipoproteins. The net effect is represented by the capacity of this pathogen to circumvent the antimicrobial functions of macrophages.
Importantly, immunostimulatory lipoproteins with little effect on MAPKp38 activation could be used to attempt the synthesis of unit-particle vaccines using nanotechnology approaches.

In conclusion, we showed that specific *M. paratuberculosis* recombinant proteins induce expression of IL-10 by macrophages whereas others are involved in preserving mycobacteria within macrophages as well as activating the MAPKp38 pathway. These data suggest that *M. paratuberculosis* proteins described herein are potentially major virulence factors and that *M. paratuberculosis* somehow actively modulates the MAPKp38 signaling pathway.

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**Author Contributions**

Conceived and designed the experiments: JB CS. Performed the experiments: CS JB EL MC. Analyzed the data: JB JS CS. Contributed reagents/materials/analysis tools: JB CS. Wrote the paper: JB JS CS.

**References**

1. Harris NB, Barletta RG (2001) *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. Clin Microbiol Rev 14: 489–512. PMID: 11432810

2. Dennis MM, Antognoli MC, Garry FB, Hirst HL, Lombard JE, Gould DH, et al. (2008) Association of severity of enteric granulomatous inflammation with disseminated *Mycobacterium avium* subspecies *paratuberculosis* infection and antemortem test results for paratuberculosis in dairy cows. Veterinary Microbiology 131: 154–163. doi: 10.1016/j.vetmic.2008.02.017 PMID: 18448275

3. Raizman EA, Fetrow JP, Wells SJ (2009) Loss of income from cows shedding *Mycobacterium avium* subspecies *paratuberculosis* prior to calving compared with cows not shedding the organism on two Minnesota dairy farms. Journal of Dairy Science 92: 4929–4936. doi: 10.3168/jds.2009-2133 PMID: 19762809

4. Stabel JR, Bradner L, Robbe-Austerman S, Beltz DC (2014) Clinical disease and stage of lactation influence shedding of *Mycobacterium avium* subspecies *paratuberculosis* into milk and colostrum of naturally infected dairy cows. J Dairy Sci 97: 6296–6304. doi: 10.3168/jds.2014-8204 PMID: 25064655

5. Stabel JR (2008) Pasteurization of colostrum reduces the incidence of paratuberculosis in neonatal dairy calves. Journal of Dairy Science 91: 3600–3606. doi: 10.3168/jds.2008-1107 PMID: 18765618

6. Clemens DL, Horwitz MA (1995) Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. J Exp Med 181: 257–270. PMID: 7807006

7. Simutis FJ, Jones DE, Hostetter JM (2007) Failure of antigen-stimulated gammadelta T cells and CD4+ T cells from sensitized cattle to upregulate nitric oxide and mycobactericidal activity of autologous *Mycobacterium avium* subspp. *paratuberculosis*-infected macrophages. Vet Immunol Immunopathol 116: 1–12. PMID: 17275098

8. Zhao B, Collins MT, Czuprynski CJ (1997) Effects of gamma interferon and nitric oxide on the interaction of *Mycobacterium avium* subspp. *paratuberculosis* with bovine monocytes. Infect Immun 65: 1761–1766. PMID: 9125559

9. Hostetter JM, Steadham EM, Haynes JS, Bailey TB, Cheville NF (2002) Cytokine effects on maturation of the phagosomes containing *Mycobacteria avium* subspecies *paratuberculosis* in J774 cells. FEMS Immunol Med Microbiol 34: 127–134. PMID: 12381463

10. Weiss DJ, Evanson OA, Moritz A, Deng MQ, Abrahamson MS (2002) Differential responses of bovine macrophages to *Mycobacterium avium* subspp. *paratuberculosis* and *Mycobacterium avium* subspp. *paratuberculosis*. Infect Immun 70: 5556–5561. PMID: 12226282

11. Kuehnel MP, Goethe R, Habermann A, Mueller E, Rohde M, Griffiths G, et al. (2001) Characterization of the intracellular survival of *Mycobacterium avium* ssp. *paratuberculosis*: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. Cell Microbiol 3: 551–556. PMID: 11488816

12. Weiss DJ, Evanson OA, Souza CD (2005) Expression of interleukin-10 and suppressor of cytokine signaling-3 associated with susceptibility of cattle to infection with *Mycobacterium avium* subspp *paratuberculosis*. Am J Vet Res 66: 1114–1120. PMID: 16111147
13. Weiss DJ, Evanson OA, de Souza C, Abrahamsen MS (2005) A critical role of interleukin-10 in the response of bovine macrophages to infection by Mycobacterium avium subsp. paratuberculosis. Am J Vet Res 66: 721–726. PMID: 15900955

14. Souza CD, Evanson OA, Weiss DJ (2006) Mitogen activated protein kinase p38 pathway is an important component of the anti-inflammatory response in Mycobacterium avium subsp. paratuberculosis-infected bovine monocytes. Microb Pathog 41: 59–66. PMID: 16716561

15. Weiss DJ, Souza CD, Evanson OA, Sanders M, Rutherford M (2006) Bovine monocyte TLR2 and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate. Immunology 109: 1098–1104. PMID: 17178558

16. Khalifeh MS, Stabel JR (2004) Effects of gamma interferon, interleukin-10, and transforming growth factor beta on the survival of Mycobacterium avium subsp. paratuberculosis and Mycobacterium avium subsp. avium. J Leukoc Biol 84: 48–55. PMID: 17913973

17. Ashwell JD (2006) The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nature reviews Immunology 6: 532–540. PMID: 16799472

18. Clark IA (2007) How TNF was recognized as a key mechanism of disease. Cytokine Growth Factor Rev 18: 335–343. PMID: 17493863

19. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. (2001) Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med 345: 1098–1104. PMID: 11596589

20. Sutcliffe IC, Russell RR (1995) Lipoproteins of gram-positive bacteria. J Bacteriol 177: 1123–1128. PMID: 7868528

21. Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, et al. (1999) Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 285: 732–736. PMID: 10426995

22. Souza C, Davis WC, Eckstein TM, Sreevatsan S, Weiss DJ (2013) Mannosylated lipoarabinomannans from Mycobacterium avium subsp. paratuberculosis alters the inflammatory response by bovine macrophages and suppresses killing of Mycobacterium avium subsp. avium organisms. PLoS One 8: e75924. doi: 10.1371/journal.pone.0075924 PMID: 24098744

23. Medzhitov R, Janeway CA Jr (1997) Innate immunity: the virtues of a nonclonal system of recognition. Cell 91: 295–298. PMID: 9363937

24. Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, Golenbock DT, et al. (2001) Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of Mycobacterium tuberculosis. J Immunol 167: 910–918. PMID: 11441098

25. Rampini SK, Selchow P, Keller C, Ehlers S, Bottger EC, Sander P (2008) LspA inactivation in Mycobacterium tuberculosis results in attenuation without affecting phagosome maturation arrest. Microbiology 154: 2991–3001. doi: 10.1099/mic.0.2008/018895-5 PMID: 18832305

26. Sakhri S, Narayanan S (2013) The IpqS knockout mutant of Mycobacterium tuberculosis is attenuated in macrophages. Microbiol Res 168: 407-414. doi: 10.1016/j.micres.2013.02.007 PMID: 23562345

27. Jacobsen CN, Aasted B, Broe MK, Petersen JL (1993) Reactivities of 20 anti-human monoclonal antibodies with leucocytes from ten different animal species. Vet Immunol Immunopathol 39: 461–466. PMID: 8116221

28. Bannantine JP, Stabel JR, Bayles DO, Geisbrecht BV (2010) Characteristics of an extensive Mycobacterium avium subspecies paratuberculosis recombinant protein set. Protein Expr Purif 72: 223–233. doi: 10.1016/j.pep.2010.03.019 PMID: 20359337

29. Bowdish DM, Sakamoto K, Kim MJ, Kroos M, Mukhopadhyay S, Leifer CA, et al. (2009) MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and Mycobacterium tuberculosis. PLoS Pathog 5: e1000474. doi: 10.1371/journal.ppat.1000474 PMID: 19521507

30. Li L, Bannantine JP, Zhang Q, Amonsin A, May BJ, Alt D, et al. (2005) The complete genome sequence of Mycobacterium avium subspecies paratuberculosis. Proc Natl Acad Sci U S A 102: 12344–12349. PMID: 16116077

31. Early J, Fischer K, Bermudez LE (2011) Mycobacterium avium uses apoptotic macrophages as tools for spreading. Microb Pathog 50: 132–139. doi: 10.1016/j.micpath.2010.12.004 PMID: 21167273

32. Bannantine JP, Stabel JR (2002) Killing of Mycobacterium avium subspecies paratuberculosis within macrophages. BMC Microbiol 2: 2. PMID: 11860602

33. Leopold I, Fricke B (1997) Inhibition, reactivation, and determination of metal ions in membrane metalloproteases of bacterial origin using high-performance liquid chromatography coupled on-line with inductively coupled plasma mass spectrometry. Anal Biochem 252: 277–285. PMID: 9344414
34. Tschumi A, Nai C, Auchli Y, Hunziker P, Gehrig P, Keller P, et al. (2009) Identification of apolipoprotein N-acyltransferase (Lnt) in mycobacteria. J Biol Chem 284: 27146–27156. doi: 10.1074/jbc.M109.022715 PMID: 19661058

35. Brülle JK, Grau T, Tschumi A, Auchli Y, Burri R, Polsfuss S, et al. (2010) Cloning, expression and characterization of Mycobacterium tuberculosis lipoprotein LprF. Biochem Biophys Res Commun 391: 679–684. doi: 10.1016/j.bbrc.2009.11.120 PMID: 19944079