Alterations of microRNAs and their predicted targeting mRNAs expression in RAW264.7 macrophages infected with Omp25 mutant Brucella melitensis

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Abstract
Brucellosis is a worldwide zoonosis caused by Brucella species and represents a serious threat to both human and animal health. Omp25 is an important immunogenic and protective antigen in Brucella species; however, the functional mechanism of Omp25 in macrophages has not yet been elucidated. Here, we constructed a Brucella melitensis omp25 deletion mutant (M5-90-Δomp25) and performed microRNA (miRNA) profiling of infected RAW264.7 cells. Eight differentially expressed miRNAs (mmu-miR-146a-5p, mmu-miR-155-5p, mmu-miR-3473a, mmu-miR-149-3p, mmu-miR-671-5p, mmu-miR-1224-5p, mmu-miR-1895, and mmu-miR-5126) were identified, with quantitative real-time PCR (qRT-PCR) analysis confirming the up-regulation of mmu-miR-146a-5p and mmu-miR-155-5p and down-regulation of mmu-miR-149-3p and mmu-miR-5126. mRNA profiling of B. melitensis M5-90-Δomp25-infected RAW264.7 cells identified 967 differentially expressed genes (DEGs) (fold change ≥ 2). Among these, we focused on genes that were predicted by TargetScan, miRanda, and PicTar to be the potential targets of the differentially expressed miRNAs. The results suggested that 17 separate genes are potentially targeted by mmu-miR-149-3p, with one of these genes, Tbr1, also targeted by mmu-miR-5126. qRT-PCR analysis confirmed the up-regulation of nine of the predicted target genes. Our findings provide important information about the functional molecules in host cells, including miRNA and their target genes, affected by Omp25 from Brucella. This information is particularly valuable for the prophylaxis and treatment of brucellosis.

Keywords
Brucella, Omp25, miRNAs, target genes, mRNAs

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Introduction
Brucellosis is a worldwide zoonosis and serious threat to the health of humans and animals alike. Brucellosis is caused by Brucella species, including B. abortus.
three major proteins, Omp25, Omp31, and Omp2b, in macrophage and cytokine cells. In another study, miRNA intracellular growth of the pathogen via regulation of down-regulation of mutants is attenuated, while Manterola et al. showed cells, with the bacterial cell outer membrane (OM) play an important role in the secretion of TNF- mediated and humoral immunity in humans and plays an important role in the secretion of TNF-α and IFN-α in macrophage and cytokine cells.

To develop efficient vaccines against brucellosis, the relationship between Omp25 and virulence has been the focus of much research. However, the results have been contradictory. Edmonds et al. reported that the virulence of B. melitensis and B. ovis omp25-deficient mutants is attenuated, while Manterola et al. showed that the absence of omp25 does not result in attenuation of virulence of B. abortus. These findings suggest that the interaction between Omp25 and host cells is very complex.

MicroRNAs (miRNAs) are noncoding RNAs approximately 22 bp in length that function as a sequence-specific, post-transcriptional regulatory mechanism. Multiple reports indicate that miRNAs play important roles in the Brucella infection process. For example, mir-125b-5p is down-regulated during B. abortus infection, which enhances the expression of its target gene, A20. In addition, B. abortus-induced down-regulation of mir-125b-5p is linked to enhanced intracellular growth of the pathogen via regulation of macrophage activation. In another study, miRNA array and quantitative real-time PCR (qRT-PCR) analyses were performed using peripheral blood mononuclear cell samples from patients with acute or chronic brucellosis to identify differentially expressed miRNAs. The assays identified four miRNAs expressed in the chronic group that were not expressed in the acute and control groups. Among these, the expression of mir-128-3p was increased while that of mir-494, mir-6069, and mir-139-3p was decreased. Further, an array-based miRNA screen using CD8+ T-cells from patients with acute or chronic brucellosis indicated that the differentially expressed miRNAs mir-126-5p and mir-4753-3p, along with their predicted target genes, are involved in the MAPK signaling pathway, cytokine–cytokine receptor interactions, endocytosis, regulation of the actin cytoskeleton, and focal adhesion.

In the current study, we constructed an Omp25-deficient B. melitensis strain (M5-90-Δomp25) to identify functional molecules in macrophages, including miRNAs and their target genes, which are affected by Brucella Omp25. miRNA and mRNA profiling conducted in RAW264.7 cells infected with B. melitensis M5-90-Δomp25 identified four differentially expressed miRNAs along with 10 potential target genes that are differentially expressed resulting of their interaction with Omp25.

**Materials and methods**

**Cells and bacterial infection**

RAW264.7 macrophages were grown at 37°C and 5% CO₂ in DMEM (Invitrogen, Carlsbad, CA). B. melitensis strain M5-90 was grown in Rubella broth medium (BD Biosciences, Franklin Lakes, NJ) for 3 d at 37°C prior to use. For infection studies, RAW264.7 cells at a density of 1 × 10⁶ cells/well (in six-well plates) were infected with the bacterial inoculums at a multiplicity of infection of 10 and incubated for 4 h. Following infection, cells were washed three times with phosphate-buffered saline (PBS) to remove extracellular bacteria.

**Preparation of the omp25 B. melitensis deletion mutant and its use in infection assays**

Three pairs of primers, Omp25-C-F/Omp25-C-R and Omp25-N-F/Omp25-N-R and Kana'-F/Kana'-R (Table 1), were designed to amplify the flanking regions of the omp25 gene from the genome of B. melitensis M5-90 as well as a kanamycin-resistance gene fragment from pEGFP-N1, respectively. The three fragments were separately ligated into vector pMD20-T, generating recombinant plasmids pMD20-T-Omp25-C, pMD20-T-Omp25-N, and pMD20-T-Kana'. pMD20-T-Omp25-C was then digested with SacI and BamHI, pMD20-T-Kana' with BamHI and KpnI, and pMD20-T-Omp25-N with KpnI and ApaI. The pGEM-7ZF suicide plasmid was also digested with the same enzymes to produce corresponding sticky ends. The digested suicide plasmid was then connected with the omp25 upstream region, the Kana' gene fragment, and the omp25 downstream region in a stepwise manner and then confirmed by sequencing. The correct recombinant plasmid was then transformed into electrocompetent B. melitensis M5-90 cells and recombinants were selected on medium containing kanamycin and ampicillin. PCR amplification using the Kana'-F and Kana'-R primers was performed to confirm the recombination events. The obtained strain was named B. melitensis M5-90-Δomp25. For infection assays, RAW264.7 cells were infected with either B. melitensis M5-90 or B. melitensis M5-90-Δomp25 cells and incubated for 4 h, as described previously.

**miRNA array screening and qRT-PCR validation**

miRNA was extracted from uninfected RAW264.7 cells and from RAW264.7 cells infected with either B. melitensis M5-90 or B. melitensis M5-90-Δomp25 as described previously. miRNA microarray analyses
were carried out by LC Sciences (Houston, TX). Using miRNA-specific primers obtained from Invitrogen, qRT-PCR analysis was performed to validate the differentially expressed miRNAs identified in the array-based screen, as described previously.\textsuperscript{12,13} U6 snRNA was used as an internal control. Each assay from different RNA sample was repeated three times.

**mRNA array screening**

High-quality total RNA was extracted from RAW264.7 cells infected with \textit{B. melitensis} M5-90 or \textit{B. melitensis} M5-90-\textit{omp}25 as described previously.\textsuperscript{12} Gene expression profiling and analysis was performed using the Agilent platform (V2, G4846A/26655) by LC-BIO Co. (China). Significantly differentially expressed genes (DEGs) (fold change $\geq 2$) were selected for further miRNA–mRNA conjoint analysis.

**miRNA–mRNA conjoint analysis**

TargetScan, miRanda, and PicTar were used to predict the target genes of the validated differentially expressed miRNAs as previously described.\textsuperscript{13} Of the potential target genes predicted by TargetScan, those with a context, score, and percentile lower than 50 were removed. Using miRanda, potential target genes with a Max Energy higher than $-10$ were removed, while potential target genes predicted using PicTar with a dideoxynucleosine score higher than $-5$ were also removed. The three lists were then collated, and genes appearing on all three lists were selected as the predicted target genes of the validated differentially expressed miRNAs.

Further comparison of the prediction-based gene list and the previous array-based gene list was performed, and the overlapping target genes were further validated by qRT-PCR analysis.

**qRT-PCR validation of miRNA–mRNA conjoint analysis results**

qRT-PCR was performed to confirm the DEGs obtained from the miRNA–mRNA conjoint analysis as previously described,\textsuperscript{12,13} using the specific primers described in Table 2. GAPDH was used as an internal control. Each RNA sample was examined in triplicate.

**Statistical analysis**

A Student’s $t$-test or one-way ANOVA was used to compare the data from the different treatment groups. $P < 0.05$ was considered statistically significant. $P < 0.01$ was considered very statistically significant.

**Results**

**Differentially expressed miRNAs in \textit{B. melitensis} M5-90-\textit{omp}25- infected RAW264.7 cells**

RAW264.7 cells were infected with \textit{B. melitensis} M5-90 or \textit{B. melitensis} M5-90-\textit{omp}25 and then examined after incubation for 4 h. Initial microarray-based screening identified eight differentially expressed miRNAs (Figure 1 and Table 3). Of these, the expression of \textit{mmu-miR-146a-5p}, \textit{mmu-miR-155-5p}, and \textit{mmu-miR-3473a} was up-regulated, while expression of \textit{mmu-miR-149-3p}, \textit{mmu-miR-671-5p}, \textit{mmu-miR-1224-5p}, \textit{mmu-miR-1895}, and \textit{mmu-miR-5126} was down-regulated. The results of further qRT-PCR validation experiments confirmed that \textit{mmu-miR-146-a-5p} and \textit{mmu-miR-155-5p} were up-regulated and that \textit{mmu-miR-149-3p} and \textit{mmu-miR-5126} were down-regulated (Figure 2), and that the strategy of initial array-based screening and further qRT-PCR validation was feasible. However, four of the initial differentially expressed

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**Table 1. Primers for constructing \textit{B. melitensis} M5-90-\textit{omp}25.**

| Primers | Sequences (5’-3’) | Inserted enzyme |
|---------|-------------------|-----------------|
| OMP25-C-F | GCGCGAGCTCTTATAGCATAATTGGACACGG | Sac I |
| OMP25-C-R | CGCGGGATCCTAAAAAGTGAAATATCCG GCC | BamHI |
| Kan’-F | GGCGGCCTGGATGGATTAGGACACGG | BamHI |
| Kan’-R | CGCGCTGAGTCAGAAGAACGCTGCAAG | Kpn I |
| OMP25-N-F | CGGGGATCCCTAAACCTGGCGTGTCGAA | Kpn I |
| OMP25-N-R | CGGGGCCCCTGCTTTCTTACAAAT | Apa I |
| OMP25-F | CGGAGATCGATGCGGAGATCTTAAGTCTC | EcoR I |
| OMP25-R | GCGCCCTGAGGAACCTGTAGGCCGATGC | Xho I |
| OMP25-C-J-F | CCGTGATATTGCTGAAGAGC | |
| OMP25-C-J-R | ATTACCTCAGAAGATCCG |
| OMP25-N-J-F | TCGGCTCCACAAAGCCG | |
| OMP25-N-J-R | GACAGGTCGCTTGTGGAAA | |
| Amp’-F | ATGAGATTCACCATTCGCC | |
| Amp’-R | TTACCAATGCTTAATCAGTG | |
miRNAs were not confirmed by qRT-PCR, suggesting the presence of false-positive signals in the array-based screen.

**Differentially expressed mRNAs in B. melitensis M5-90-omp25-infected RAW264.7 cells**

As shown in Figure 3 and Supplementary Figure 1, the mRNA microarray identified 967 DEGs in RAW264.7 cells infected with *B. melitensis* M5-90-omp25. Further miRNA-mRNA conjoint analysis indicated that among the 967 DEGs, 17 genes, namely *Slc7a119* (also known as *xCT*), *Mras* (muscle RAS oncogene homolog), *Slc31a2* (solute carrier family 31 member 2), *Asxl3* (additional sex combs-like 3), *Ifit1* (interferon-induced with tetratricopeptide repeats), *Obox6* (oocyte-specific homeobox 6), *Cdk1* (cyclin-dependent kinase 1), *Ceacam14* (CEA-related cell adhesion molecule 14), *Dusp16* (dual-specificity phosphatase 16), *Igfbp2* (insulin-like growth factor-binding protein 2), *Ikbke* (inhibitor of kappaB kinase epsilon), *Olr1* (oxidized low density lipoprotein (lectin-like) receptor 1), and *Tbr1* (neuroD1 transcription factor) were differentially expressed.

| Gene symbol | GenBank ID | Primer sequence (5'-3') |
|-------------|------------|-------------------------|
| Bcl6b       | NM_007528  | F: AAGAGCGCAAAAGGAGCTTC  |
|             |            | R: CCCCCGGAAAATTGAAATGAAAG |
| Prss46      | NM_183103  | F: CCCCTCTCAGCCAGCAATCT  |
|             |            | R: TGTTCTCAATGCTTGGCTTT |
| Olr1        | NM_138648  | F: CTTCCATGCGCCCTTTCAGC |
|             |            | R: TGCCACCCAAAGATTGG |
| Ikbke       | NM_019777  | F: CGGCTCAGCACGCTTTCCTCC |
|             |            | R: GGCGTGATGATGACATGAT |
| Ceacam14    | NM_025957  | F: TGCTCAAGCAGTCCCCCTTTT |
|             |            | R: TTTGTGATGGACTGCGGAGT |
| Slc7a11     | NM_011990  | F: GCACCTAAGCCTCTTCAAGAC |
|             |            | R: GATAGCTGCATGTATTTCTTGTAAG |
| Asxl3       | NM_00116777 | F: TCTGAATGCTGAGCTTACACT |
|             |            | R: CAACGACTAGATCAATTGGTCC |
| Mrsas       | NM_008624  | F: CAATGCCAGATACCTGAAATGG |
|             |            | R: CGCTCCACAGCAACACTG |
| Slc31a2     | NM_025286  | F: TTTGAGATGAAACCAGAAATGG |
|             |            | R: GGAGGGAGGTAGCTTCAAG |
| Cdk1        | NM_007659  | F: GGACGAGAACGCTGTTGAT |
|             |            | R: GAGATTCGTTTGGCAGGATCA |
| Ifit1       | NM_001025602 | F: ACACGACAGAAGGACTTTG |
|             |            | R: ACTGCCCTCGTAACTGTCAA |
| Nos2        | NM_010927  | F: GCCCCACGGGACACAGT |
|             |            | R: AACAGTCACTGCCCTCCTAAC |
| Ifit1       | NM_008331  | F: GCCAAGTCTGAGAGGACTTG |
|             |            | R: TCCCATGGTTGCTGAAATGG |
| Dusp16      | NM_130447  | F: TCCTGGATGTTGTATCTTCT |
|             |            | R: CCTGCTGACGTAGTGAATT |
| Obox6       | NM_145710  | F: GCACCTCCTGAGTCTGTCG |
|             |            | R: GGATGTGGGGCTGTGAGT |
| Tbr1        | NM_009322  | F: GATCCCAATCAGCTGGTTT |
|             |            | R: GTGTCCCGAGCCACAGA |
| Igfbp2      | NM_008342  | F: GCCCCTGGAACATCTCTACT |
|             |            | R: TACGCGCATGCTGGCTCA |
| GAPDH       | NM_008084.3 | F: TGTTGGCGTCTCTGATGCA |
|             |            | R: CCTGCTCACCACCCCTCTTGA |

**Table 2.** Primers of qRT-PCR identification for the DEGs (fold change >2) obtained from miRNAs–mRNA conjoint analysis.
Prss46 (proteases, serine), Tbr1 (T-box brain 1), Bcl6b (B cell CLL/lymphoma 6, member B), Il1rl1 (IL-1 receptor-like 1), and Nos2 (NO synthase 2, inducible), were predicted to be target genes of mmu-miR-149-3p by the bioinformatics analyses, and were up-regulated. One of these 17 genes, Tbr1, was also predicted to be a target of two further miRNAs, mmu-miR-149-3p and mmu-miR-5126, and to be up-regulated in both cases (Table 4, Figures 4 and 5).

After specific primers were designed (Table 2), qRT-PCR validation of the predicted target genes was performed. The results confirmed the up-regulation of Bcl6b, Nos2, Ikbke, Slc31a2, Dusp16, Ifit1, Slc7a11, II1rl1, and Olr1 (Figure 6).

Discussion

Brucella species are Gram-negative, intracellular bacteria responsible for the zoonosis brucellosis. To survive within host cells, Brucella species affect the secretion of cytokines, including TNF-α, and induce dysregulation of the normal protective function of the
immune response. During the infection process, Omp25 on the surface of Brucella cells controls TNF-α production in human macrophages. Functional insight into Omp25 would aid in elucidating the virulence mechanisms of Brucella. B. melitensis vaccine strain M5-90 has some residual virulence and may result in abortion in pregnant sheep. In the current study, we constructed Omp25-deficient strain B. melitensis M5-90-\(\Delta\)omp25 to examine the function of Omp25.

Table 4. mRNA array-based expression levels of DEGs (fold change ≥ 2) obtained from miRNAs-mRNA conjoint analysis.

| Gene symbol | P-value     | Mean (G2/G1) | Log2 (G2/G1) |
|-------------|-------------|--------------|--------------|
| Bcl6b       | 1.85E-02    | 27.61        | 74.78        | 1.44 |
| Prss46      | 3.61E-02    | 84.56        | 185.70       | 1.13 |
| Olr1        | 3.83E-02    | 61.37        | 137.79       | 1.17 |
| Ikbke       | 3.69E-03    | 54.41        | 135.77       | 1.32 |
| Ceacam14    | 1.49E-03    | 20.08        | 50.91        | 1.34 |
| Slc7a11     | 8.17E-03    | 35.95        | 161.74       | 2.17 |
| Asxl3       | 8.10E-03    | 6.39         | 20.20        | 1.66 |
| Mras        | 1.49E-02    | 25.39        | 75.84        | 1.58 |
| Slc31a2     | 9.61E-03    | 271.65       | 991.51       | 1.87 |
| Cdk1        | 3.13E-03    | 47.74        | 115.75       | 1.28 |
| Il1r1       | 1.75E-04    | 642.66       | 1461.13      | 1.18 |
| Nos2        | 1.31E-03    | 114.00       | 869.50       | 2.93 |
| Ifit1       | 2.94E-03    | 545.90       | 1548.83      | 1.50 |
| Dusp16      | 2.82E-03    | 253.67       | 627.60       | 1.31 |
| Obox6       | 2.87E-03    | 6.39         | 20.75        | 1.70 |
| Tbr1        | 3.78E-02    | 26.10        | 71.27        | 1.45 |
| Igfbp2      | 1.58E-02    | 19.69        | 39.67        | 1.01 |

Figure 4. The validated differentially expressed miRNAs and their predicted targeting genes in RAW264.7 infected with Omp25 mutant B. melitensis.

Figure 5. Heat map of the DEGs selected from mRNA array screening in RAW264.7 infected with Omp25 mutant B. melitensis (fold change ≥ 2) (P < 0.05). These DEGs are also the predicted targeting genes of the validated differentially expressed miRNAs. The experiments in which RAW264.7 were infected with B. melitensis M5-90 was repeated three times. M5-90-1, M5-90-2, and M5-90-3 was the sample of each infection assay, respectively. Red denotes induction and green denotes suppression.

Figure 6. qRT-PCR validation for the identified up-regulated DEGs in RAW264.7-M-\(\Delta\) Omp25.
To improve the signal/noise ratio when identifying DEGs in RAW264.7 cells infected with \( B.\ melitensis \) M5-90-\( \Delta \)omp25, we used a research strategy that generated two gene lists from different analysis methods. After differentially expressed miRNAs were identified, their target genes were predicted using different bioinformatics software tools, and these target genes formed the first gene list. The second gene list was obtained from mRNA array-based screening. The overlapping genes from the two gene lists formed the final list of target genes. qRT-PCR analysis was performed to validate the DEGs on the final list. Among these overlapping genes, nine genes, all of which are targets of \( \text{mmu-miR-149-3p} \), were identified as the DEGs of RAW264.7 cells infected with \( B.\ melitensis \) M5-90-\( \Delta \)omp25. The results of qRT-PCR validation demonstrated that the strategy is feasible and effective.

\( Bcl6b, \ Nos2, \ Ikbke, \ Slc31a2, \ Dusp16, \ Ifit1, \ Slc7a11, \ Il1r1l, \) and \( Olr1 \) were confirmed as the target genes of \( \text{mmu-miR-149-3p} \). In humans, \( BCL6b \) is expressed in a small subset of Ag-experienced CD8\(^+\) T-cells and is required for the enhanced secondary response of memory CD8\(^+\) T-cells,\(^16\) while \( NOS2 \) is involved in the production of NO. A cellular defense mechanism against \( Brucella \) infection.\(^17\) DUSPs are a family of protein phosphatases.\(^18\) In macrophages, \( Dusp16 \) RNA expression is inducible by TLR stimulation.\(^19,20\) Dusp16 preferentially dephosphorylates c-Jun N-terminal kinase- and p38-family MAPKs.\(^21\) In infected macrophages, to suppress host immune responses for intracellular survival, \( Mycobacterium tuberculosis \) EIs (enhanced intracellular survival protein) initiates the inhibition of autophagy and phagosome maturation by \( N^+ \)-acetylating Lys55 of DUSP16.\(^22\) IFIT proteins are critical mediators of mammalian innate antiviral immunity, with IFIT1 proving to be a potent inhibitor of alphavirus replication.\(^23\) In the mouse lung, intraperitoneal challenge with LPS induces the up-regulation of \( Olr1 \) expression, suggesting that \( Olr1 \) is involved in LPS-induced lung inflammation.\(^24\) The up-regulation of all of these genes in our infection model indicates that the functional mechanism of Omp25 might involve multiple signaling pathways and a large number of signaling molecules.

This is the first report describing miRNA expression analysis of macrophage cells infected with an Omp25-deficient \( Brucella \) strain. Although further efforts are needed to fully elucidate the interaction between Omp25 and these molecules, our findings provide some important information that will aid in the prophylaxis and treatment of brucellosis.

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