CD14 gene silencing alters the microRNA expression profile of RAW264.7 cells stimulated by *Brucella melitensis* infection

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Abstract

Innate recognition of *Brucella* spp. is a key step in the activation of inflammation. CD14 binds PAMPs and is involved in LPS-induced pro-inflammatory cytokine release. Previously we showed that knock down of CD14 in RAW264.7 macrophages disrupted *Brucella*–host interactions. However, its effect on the macrophage microRNA (miRNA) expression profile, especially after stimulation by *Brucella* infection, is still unclear. To identify miRNAs involved in the macrophage response to *Brucella* infection, we performed miRNA expression profiling of CD14 knock-down RAW264.7 (224.3) macrophages infected with *Brucella melitensis*, and demonstrated, for the first time, that CD14 knock down significantly up-regulated the expression of mmu-miR-199a-3p and mmu-miR-183-5p in these conditions. These miRNAs have a well-characterized association with the target genes involved in immune response, inflammatory response, innate immune response, apoptosis processes, anti-apoptosis, cytokine production and cytokine-mediated signaling pathways. Among the 104 inflammation-related candidate target genes of mmu-miR-199a-3p and mmu-miR-183-5p in the 224.3$^+$ *B. melitensis* group cells, the expression of the *Cbl-b*, a potential target of mmu-miR-199a-3p, was confirmed to be down-regulated using qRT-PCR and Western blot analysis. Our findings suggest that CD14 functions in the *Brucella*–host interaction may be through altered miRNA expression, and regulation of Cbl-b proteins.

Introduction

Brucellosis is an important zoonosis throughout the world. *Brucella* spp., the pathogen responsible for brucellosis, survive and replicate predominantly in macrophages. LPS is one of the PAMPs and the key virulence determinant of *Brucella* spp.[1] CD14 is present as a membrane-bound receptor in myeloid cell lines or in a soluble form in serum. When CD14 binds LPS, CD14–LPS complex binds to TLR4 and triggers the release of pro-inflammatory cytokines.[2–5] Our previous study suggested that lentivirus-mediated RNAi targeting mCD14 inhibits TNF-α secretion, iNOS expression, and NO production in RAW264.7 cells stimulated by *B. melitensis in vitro*.[6]

MicroRNAs (miRNAs), 18–25 nucleotide non-coding RNAs, post-transcriptionally regulate gene

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expression. miRNAs play important roles in *Brucella* infections.[7–9] It has been reported that a high-through-put sequencing approach resulted in identification of 57 miRNAs that were differentially expressed between mock- and *Brucella*-infected RAW264.7 cells.[10] In macrophages, *Brucella abortus* infection leads to down-regulation of miR-125b-5p. miR-125b-5p targets A20, an inhibitor of NF-kB activation. In the presence of miR-125b-5p, *B. abortus* survival is attenuated.[11] A miRNA array-based initial screen followed by quan-titative reverse transcription PCR (qRT-PCR) validation was used to identify the differentially expressed miRNAs in PBMCs of patients with acute or chronic brucellosis and healthy controls. The results indicated that the expression of miR-1238-3p was increased while expres-sion of miR-494, miR-6069 and miR-139-3p were decreased. Thus, these miRNAs have the potential to be markers for chronic cases.[12] Using the same strategy, the differentially expressed miRNAs were identified in CD8$^+$ T cells of patients with acute or chronic brucel-losis and healthy controls. miR-126-5p and miR-4753-3p were decreased, and are therefore thought to have the potential to be regulators of CD8$^+$ T-cell-related marker genes for chronic brucellosis infections.[13] However, in the presence of *Brucella melitensis* infection, the effects of mCD14 on the miRNA expression profile is unclear.

In this study, to identify miRNAs affected by *Brucella* spp. infection in the absence CD14, we ana-lyzed the miRNA expression profiles of mCD14 knockdown RAW264.7 cells challenged with *B. meli-tensis* M5-90 using a miRNA array. We observed up-regulation of mmu-miR-199a-3p and mmu-miR-183-5p, and confirmed this by qRT-PCR. Interestingly, among the predicted target genes of mmu-miR-199a-3p and mmu-miR-183-5p, the signifi-cantly enriched gene ontology (GO) terms were apop-tosis process, immune response, inflammatory response, innate immune response, anti-apoptosis, cytokine production and cytokine-mediated signaling pathway. Among the 104 candidate target genes, the expression of the *Cbl-b* gene in mCD14 knock-down RAW264.7 cells challenged with *B. melitensis* M5-90 was validated to be down-regulated.

Materials and methods

Cell culture and Brucella melitensis *infection*

The RAW264.7 cell line, used as a control, was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China), and routinely grown, as previously described.[6] The 224.3 cell line, which is a mCD14 knock-down RAW264.7 cells line, and NC, which is a RAW264.7 stable cell line carrying shRNA-NC, were established and identified in our pre-vious study.[6] The same number of 224.3, RAW264.7 and NC cells (5×10^5 cells per well) were infected

with *B. melitensis* M5-90 for 6 h, as previously described.[6]

miRNA array profiling and analysis

RNA preparation and quantitation were performed as previously described.[14] The small RNAs were extracted from the 224.3, RAW264.7 and NC cells, after infection with *B. melitensis* M5-90, and miRNA microarray ana-lyses were undertaken by LC Sciences as previously described.[14]

qRT –PCR for miRNAs

To validate the differential expression, as previously described,[14] small RNAs were extracted from the cells and qRT-PCR was performed to determine miRNA expression levels.

Target gene prediction and GO

To predict targets of significantly differentially expressed miRNAs, TargetScan, PicTar, miRDB and microRNA.org were used. GO (http://www.geneontol-ogy.org/) assignments were conducted as previously described.[14]

qRT-PCR for mRNAs

To measure mRNA levels, high-quality total RNA was isolated from the 224.3, RAW264.7 and NC cells, after infection with *B. melitensis* M5-90, and subjected to qRT-PCR as previously described.[6]

Western blot analysis

The total proteins were harvested from the 224.3, RAW264.7, and NC cells, after infection with *B. meli-tensis* M5-90, and Western blot analysis was performed as described in a previous study.[6] Primary Abs included Cbl-b (G-1) mouse monoclonal IgG$_1$ (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH rabbit polyclonal IgG (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA). Secondary Abs used were HRP-labeled goat anti-mouse IgG (1:4000 dilution; Zymed Laboratories, San Francisco, CA, USA) and HRP-labeled goat anti-rabbit IgG (1:3000 dilution; Santa Cruz Biotechnology).

Data analysis

The Student's *t*-test and one-way ANOVA were per-formed for data analysis as previously described.[6] A *P*-value of < 0.05 was considered significantly differ-ent and a *P*-value of < 0.01 was considered very significant.

Results

Brucella melitensis-induced differential miRNA levels in mCD14 knockdown RAW264.7 cells

RNA samples obtained from 224.3, RAW264.7 and NC stimulated by *B. melitensis* infection were compared using microarray analysis. Figure 1(A) shows the relative expression of the 15 differentially expressed genes, namely mmu-miR-5105, mmu-miR-199a-3p, mmu-miR-762, mmu-miR-5109, mmu-miR-146b-5p, mmu-miR-5130, mmu-miR-191-5p, mmu-miR-1894-3p, mmu-miR-690, mmu-miR-183-5p, mmu-miR-5115, mmu-miR-5126, mmu-let-7i-5p, mmu-miR-181b-5p and mmu-miR-361-5p. Among them, three miRNAs (mmu-miR-199a-3p, mmu-miR-183-5p and mmu-miR-361-5p) were up-regulated and 10 miRNAs (mmu-miR-5105, mmu-miR-762, mmu-miR-5109,

mmu-miR-146b-5p, mmu-miR-5130, mmu-miR-191-5p, mmu-miR-1894-3p, mmu-miR-690, mmu-miR-5115 and mmu-miR-5126) were down-regulated in the 224.3$^+$ *B. melitensis* group compared with the control RAW264.7$^+$ *B. melitensis* group and NC + *B. melitensis* group. Because of the unreasonable data obtained from the array, two miRNAs (mmu-let-7i-5p, mmu-miR-181b-5p) were considered noise signals (Table 1).

To verify the data from the initial array-based screen, qRT-PCR experiments were performed for mmu-miR-199a-3p and mmu-miR-183-5p, as these had been reported previously.[6] U6 snRNA abundance was used as an internal control. As shown in Figure 1(B), the up-regulation of both mmu-miR-199a-3p and mmu-miR-183-5p was confirmed in the 224.3$^+$ *B. melitensis* group.

Target gene prediction and GO

To dissect the mechanisms through which CD14 affects the host cells through regulation of miRNAs expression in the *B. melitensis* infection more clearly, we predicted the potential targets of mmu-miR-199a-3p and mmu-miR-183-5p using TargetScan, PicTar, miRDB and microRNA.org. In total, 104 candidate genes were identified by at least one of the four different algorithms (Table 2). To evaluate the function of the predicted target genes, we analyzed the frequency of specific GO terms among the predicted target genes. Significantly enriched GO terms in the predicted target genes of mmu-miR-199a-3p and mmu-miR-183-5p were apoptosis process, immune response, inflammatory response,
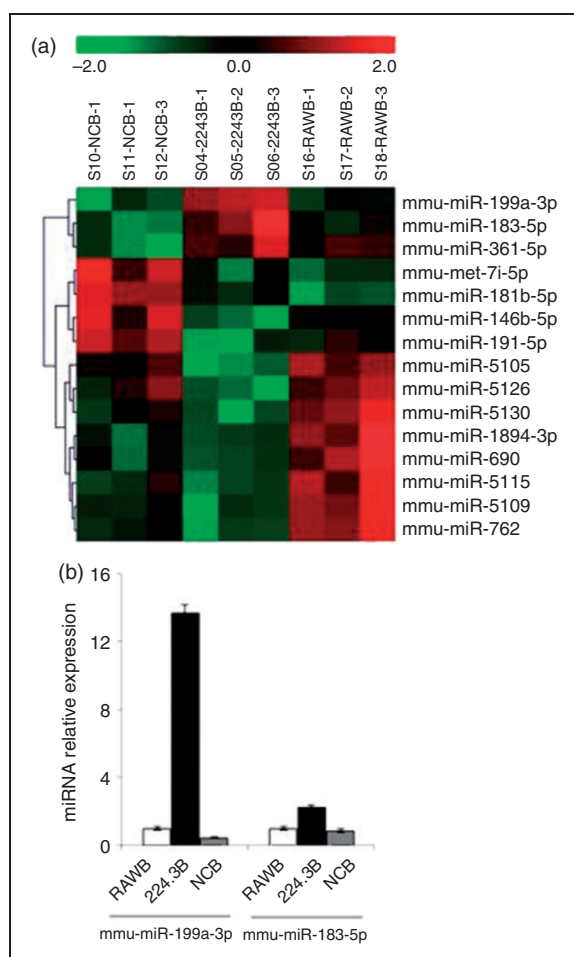

Figure 1. Up-regulation of mmu-miR-199a-3p and mmu-miR-183-5p expression. (a) Heat-map representation of data from two-way cluster analysis of differentially expressed miRNAs ($P < 0.05$) measured by an array-based screen of 224.3, RAW264.7 and NC cells, which are stimulated by *B. melitensis* infection. Green indicates suppression and red indicates induction compared with in control cells. (b) Validation of mmu-miR-199a-3p and mmu-miR-183-5p up-regulation by qRT-PCR.

Table 1. The significantly differentially expressed miRNAs by microarray analysis.

Reporter name	P-Value	Group 1 RAWB mean	Group 2 NCB mean	Group 3 224.3B mean
mmu-miR-5105	2.74E-04	1704	1166	439
mmu-miR-199a-3	5.49E-04	36	13	1240
mmu-miR-762	6.52E-04	10,409	4691	3374
mmu-miR-5109	2.44E-03	11,431	5507	4154
mmu-miR-146b-5	2.58E-03	2149	4564	1176
mmu-miR-5130	3.07E-03	2123	790	379
mmu-miR-191-5p	4.76E-03	2956	4846	1833
mmu-miR-1894-3	4.77E-03	1362	385	329
mmu-miR-690	6.74E-03	5084	3096	2802
mmu-miR-183-5p	7.43E-03	177	90	527
mmu-miR-5115	7.66E-03	1129	482	329
mmu-miR-5126	7.73E-03	21,076	18,523	13,043
mmu-let-7i-5p	8.46E-03	2458	4357	2587
mmu-miR-181b-5	3.47E-04	49	110	66
mmu-miR-361-5p	7.81E-03	270	134	346

Table 2. Inflammation-related candidate target genes of mmu-miR-199a-3p and mmu-miR-183-5p.

Gene	miRNA	miRNA base
Apoptosis process **GO: 0006915**		
Psen2	mmu-miR-183-5p	A, B, C
Pdcd4	mmu-miR-183-5p	A, B, C
Bnip3l	mmu-miR-183-5p	A, B, C
Csnk2a1	mmu-miR-183-5p	A, B, C
Vdac1	mmu-miR-183-5p	A, B
Mef2c	mmu-miR-183-5p	A, C
Prkca	mmu-miR-183-5p	B, C
Pim1	mmu-miR-183-5p	B, C
Rhob	mmu-miR-183-5p	B, C
Pdcd6	mmu-miR-183-5p	A
Stk17b	mmu-miR-183-5p	A
Birc6	mmu-miR-183-5p	B
Taok1	mmu-miR-183-5p mmu-miR-199a-3p	A, C
Prune2	mmu-miR-183-5p	C
Sh3glb1	mmu-miR-199a-3p	A, C, D
Pak4	mmu-miR-199a-3p	A, C, D
Rnf216	mmu-miR-199a-3p	A, C, D
Gja1	mmu-miR-199a-3p	B, D
Acvr1c	mmu-miR-199a-3p	C, D
Pawr	mmu-miR-199a-3p	C, D
Sema3a	mmu-miR-199a-3p	C, D
Tns4	mmu-miR-199a-3p	A
Bnipl	mmu-miR-199a-3p	A
App	mmu-miR-199a-3p	B
Igsf4a	mmu-miR-199a-3p	B
Phlda1	mmu-miR-199a-3p	B
Bbc3	mmu-miR-199a-3p	B
Ece1	mmu-miR-199a-3p	B
Pura	mmu-miR-199a-3p	B
Dido1	mmu-miR-199a-3p	B
Erbb4	mmu-miR-199a-3p	C
Map3k5	mmu-miR-199a-3p	C
Ddit4	mmu-miR-199a-3p	C
Rabep1	mmu-miR-199a-3p	C
Fasl	mmu-miR-199a-3p	D
Naip5	mmu-miR-199a-3p	D
Casp12	mmu-miR-199a-3p	D
Birc3	mmu-miR-199a-3p	D
Birc2	mmu-miR-199a-3p	D
Luc7l3	mmu-miR-199a-3p	D
Sgms1	mmu-miR-199a-3p	D
Zfp110	mmu-miR-199a-3p	D
Tax1bp1	mmu-miR-199a-3p	D
Ank2	mmu-miR-199a-3p	D
Mapt	mmu-miR-199a-3p	D

(Continued)

Table 2. Continued.

Gene	miRNA	miRNA base
Apaf1	mmu-miR-199a-3p	D
Tmbim4	mmu-miR-199a-3p	D
Epha7	mmu-miR-199a-3p	D
Rock1	mmu-miR-199a-3p	D
Ppp1r13b	mmu-miR-199a-3p	D
Prune2	mmu-miR-199a-3p	D
Elmo1	mmu-miR-199a-3p	D
Cadm1	mmu-miR-199a-3p	D
Bcl2l14	mmu-miR-199a-3p	D
Immune response **GO: 0006955**		
Cx3cl1	mmu-miR-183-5p	C
Cbl-b	mmu-miR-199a-3p	A, C
Cxcl11	mmu-miR-199a-3p	A, D
Cd55	mmu-miR-199a-3p	A
Lbp	mmu-miR-199a-3p	A
Fasl	mmu-miR-199a-3p	D
Ccl7	mmu-miR-199a-3p	D
Gm106	mmu-miR-199a-3p	D
Ccl20	mmu-miR-199a-3p	D
Inflammatory response **GO: 0006954**		
Atrn	mmu-miR-183-5p	C
Cxcl11	mmu-miR-199a-3p	A, D
Cela1	mmu-miR-199a-3p	A, D
Thbs1	mmu-miR-199a-3p	A, D
Hdac9	mmu-miR-199a-3p	B, D
Ccl7	mmu-miR-199a-3p	D
Mapk8	mmu-miR-199a-3p	D
Sgms1	mmu-miR-199a-3p	D
Tnfrsf4	mmu-miR-199a-3p	D
Nlrp3	mmu-miR-199a-3p	D
Tirap	mmu-miR-199a-3p	D
Ccl20	mmu-miR-199a-3p	D
Innate immune response **GO: 0045087**		
Cd55	mmu-miR-199a-3p	A, D
Lbp	mmu-miR-199a-3p	A, D
Map3k5	mmu-miR-199a-3p	C
Tirap	mmu-miR-199a-3p	D
Ifih1	mmu-miR-199a-3p	D
Il18rap	mmu-miR-199a-3p	D
Anti-apoptosis **GO: 0006916**		
Mapk8ip1	mmu-miR-183-5p	A, B, C
Psen2	mmu-miR-183-5p	A, B
Tcf7l2	mmu-miR-183-5p	A, C
Foxo1	mmu-miR-183-5p	B, C
Birc6	mmu-miR-183-5p	B
Nr3c1	mmu-miR-183-5p	C

(Continued)

Table 2. Continued.

Gene	miRNA	miRNA base
Serbp1	mmu-miR-183-5p	C
Gclc	mmu-miR-199a-3p	A, D
Fn1	mmu-miR-199a-3p	A, C, D
Sh3glb1	mmu-miR-199a-3p	A, C, D
Ndufaf4	mmu-miR-199a-3p	A, D
Son	mmu-miR-199a-3p	B
Gsk3b	mmu-miR-199a-3p	B
IGF1	mmu-miR-199a-3p	C
NR3C1	mmu-miR-199a-3p	C
Tax1bp1	mmu-miR-199a-3p	D
Tmbim4	mmu-miR-199a-3p	D
Bdnf	mmu-miR-199a-3p	D
Nrg1	mmu-miR-199a-3p	D
Cytokine production GO: 0001816		
Txk	mmu-miR-183-5p	A
Cd226	mmu-miR-199a-3p	A, D
Cytokine-mediated signaling pathway GO: 0019221		
Gfra2	mmu-miR-199a-3p	D
Il13ra1	mmu-miR-199a-3p	D
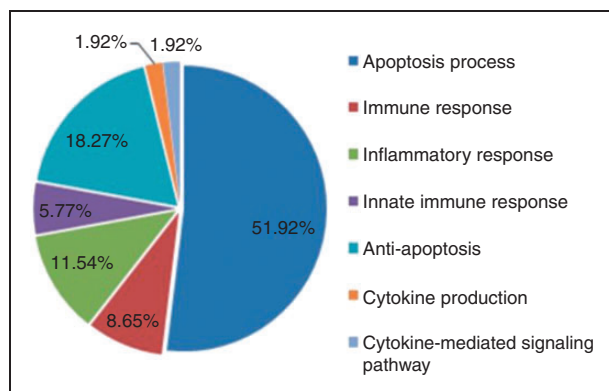
Figure 2. Significantly enriched GO terms among the predicted target genes of mmu-miR-199a3p and mmu-miR-183-5p.

innate immune response, anti-apoptosis, cytokine production and cytokine-mediated signaling pathway (Figure 2).

Down-regulation of Cbl-b in 224.3⁺ B. melitensis group cells

Among the 104 inflammation-related candidate target genes of mmu-miR-199a-3p and mmu-miR-183-5p, 31 were predicted by at least two different algorithms. Specific primers were designed and qRT-PCR was performed to determine the mRNA level of these genes (Table 3). The results showed that the mRNA level of

the *Cbl-b* genes in 224.3$^+$ *B. melitensis* group cells was down-regulated (Figure 3A). Western blot analysis results indicated that the protein expression from the *Cbl-b* genes in 224.3$^+$ *B. melitensis* group cells was also down-regulated (Figure 3B). These data suggest that *Cbl-b* might be a target of mmu-miR-199a-3p in 224.3$^+$ *B. melitensis* group cells, and that CD14 gene silencing might affect *Cbl-b* expression in RAW264.7 cells via up-regulation of mmu-miR-199a-3p after *B. melitensis* infection.

Discussion

Recent reports have provided evidence that miRNA plays an important role in bacterial infections, including *Brucella* infection.[8–13] As an important innate immune molecule, CD14 is involved in the initiation of the inflammatory response to sepsis. However, there are few reports about the relationship between CD14 and the miRNA expression profile of host cells. Here, we are the first to elucidate the role of CD14 in affecting the miRNA expression profile of macrophages after stimulation by *Brucella* infection. To dissect the mechanisms through which CD14 affects the miRNA expression profile of macrophages after *Brucella* infection, an initial throughput, array-based screen was performed, followed by qRT-PCR validation. Consistent with the values obtained from the array, up-regulation of mmu-miR-199a-3p and mmu-miR-183-5 was further confirmed with qRT-PCR.

In our study, the targets of mmu-miR-199a-3p and mmu-miR-183-5p were predicted by at least one of four different algorithms (TargetScan, PicTar, miRDB and microRNA.org). More than 1000 different candidate genes were identified (data not shown). Because *Brucella* stimulates a robust inflammatory response,[15] the significantly enriched GO terms in the predicted target genes of mmu-miR-199a-3p and mmu-miR-183-5p were focused on 104 inflammation-associated candidate genes. Among them, 31 genes predicted by at least two algorithms were selected for further experiment validation. qRT-PCR results indicated that the *Cbl-b* genes in the cells from 224.3$^+$ *B. melitensis* group were down-regulated, which was confirmed by reduced protein expression of the gene product in Western blot analysis.

Mammalian Cbl family proteins consist of c-Cbl, Cbl-b and Cbl-3. Cbl-b is an E3 ubiquitin ligase.[16–19] In the absence of co-stimulation, Cbl-b$^{-/-}$ T cells show constitutive activation that results in spontaneous autoimmunity or enhanced susceptibility to autoantigen.[20,21] Cbl-b has the capacity to regulate Fcγ receptor-mediated phagocytosis. The macrophages from Cbl-b$^{-/-}$ mice demonstrate enhanced Fcγ receptor-mediated tyrosine phosphorylation, phagocytosis and target binding.[22] Cbl-b deficiency could exaggerate high-fat diet-induced insulin resistance through

Table 3. Primers for candidate target genes of miR-199a-3p and miR-183-5p.

Gene symbol	Genbank ID	Primer sequence(5′-3′)
Prkca	NM_011101	F: ACTGCTCGTTTGGTTACTGG R: ATTCATACACGCTTGCTTCC
Rhob	NM_007483	F:AACCTCATCTACTTCTGATG R:TAAATAGACCCCAATACCTG
Mapk8ip1	NM_001202445	F:CCTGGGCCTGTTTCATGCTA R:TGGCATGGGCTTTGTCAAG
Dusp10	NM_022019	F:GTCGCTCTTCTTTCCTTTCCTT R:ACTTTCACAAACACTCCCAACC
TAOK1	NM_144825	F: ATGTTTAGAGTAAGCAGAGG R: ATAAGCCTTACCAGTTAGTG
Pawr	NM_054056	F: GGCCTACAATGCCTTATTACTAATGC R: CAGATGAGTCTTTCCGTCTAAGTGTTA
Cd226	NM_178687	F: GACCACATGGCTTTCTTGCTCT R: AAGTGTCCTTGGCAACCATCATG
Cbl-b	NM_001033238	F: TGTGGATGTTTCTTACCACTCGTT R: GTGATTATCCGAGGCATCTCTGT
Cxcl11	NM_019494	F: CGGGATGAAAGCCGTCAA R:CCGTTACTCGGGTAAATTACAGAAG
Thbs1	NM_011580	F:AGGTAGCTGAGGCGGATCAG R: TGCCTCTGCTGCCTTTCC
Cela1	NM_033612	F: AGCACAACCTGAGCCAGAATG R: TTGTTAGCCAGGATGGTTCC
Gclc	NM_010295	F: GCCACTGAATTCTGCTAGCTCTT R: CCATTTGACAAAGCTCTCAGTGTAT
Fn1	NM_010233	F: CCAACTAGGAGGAAATGTACTGAATG R:CAGGTAAGAGAAAGGTATTTCTAGGCA
Psen2	NM_011183	F: GGTGCCATCTCTGTGTACGATCT R: AGTTTCCACCAGCATCCTCAGT
Pdcd4	NM_001168491	F: GATGAGACCGCATTTGAGAA R: CTAAGGACACTGCCAACACC
Bnip3l	NM_009761	F: GCAATGCCACCAGCAGATTA R: CTGCATGGCTAGAACACAGCTT
Csnk2a1	NM_007788	F: CCATATTGTCTGTGTGAAGC R: CGTTGGTAAGACTTTGTTTC
Vdac1	NM_011694	F: TTTTAGACTGATCCTCACAC R: GGTGTAAAACAGGTTAAGTG
Mef2c	NM_025282	F: CACTAGCACTCATTTATCTC R: AAACAGGTTCTGACTTGATG
Pim1	NM_008842	F: TGTCGATGGAACTTTAGTC R: GACAGGGACTTAAATATGGT
Pak4	NM_027470	F: TTGCCAGGCAGAGGCCAGTAGA R: GAGTTCAGTAGTAGGTCCCACGAA
Rnf216	NM_080561	F: ATCCTGACAGAAGATGATTC R: TAATGACTTTCTGCTCCAAT
Gja1	NM_010288	F: ACAGTCTTTTAGATTGACGA R: GTAGGTGTCTTTAGCTTACA
Acvr1c	NM_001111030	F: TTCTGTCACAGTTCCAACAACGT R: GGTGCAGTGTGATATTGTTGCA
Sema3a	NM_001243073	F: AGTCAGCCACCGGCTATCAG R: CGGCAGTGGCGCTTAAGA
Hdac9	NM_001271386	F: CAAATGAAGCTGACGCAAATG R: CATCGCAGCATCTGATTGGA

Table 3. Continued.

| Gene symbol	Genbank ID	Primer sequence(5′-3′)
Cd55	NM_010016	F: AATACTGTTGATTGGGACGATGAG
		R: TTTGGTGGCTCTGGACAATG
Lbp	NM_008489	F: ACCTCTGACCTGCAGCCTTA
		R: GGAGAGCGGTGATTCCGAT
Tcf7l2	NM_001142924	F: AATGTTTCCTAAATCCTTGC
		R: ATTCCAAATTCAAAGACTGC
Foxo1	NM_019739	F: ACAGACAGCCCGTCAGGATAGA
		R: TAGCAATGGAACAGGAGCAAGG
Ndufaf4	NM_026742	F: CGGGAAAAGATGTGGAAGATGA
		R: TCACCTCCCATCTTTGCTGTT
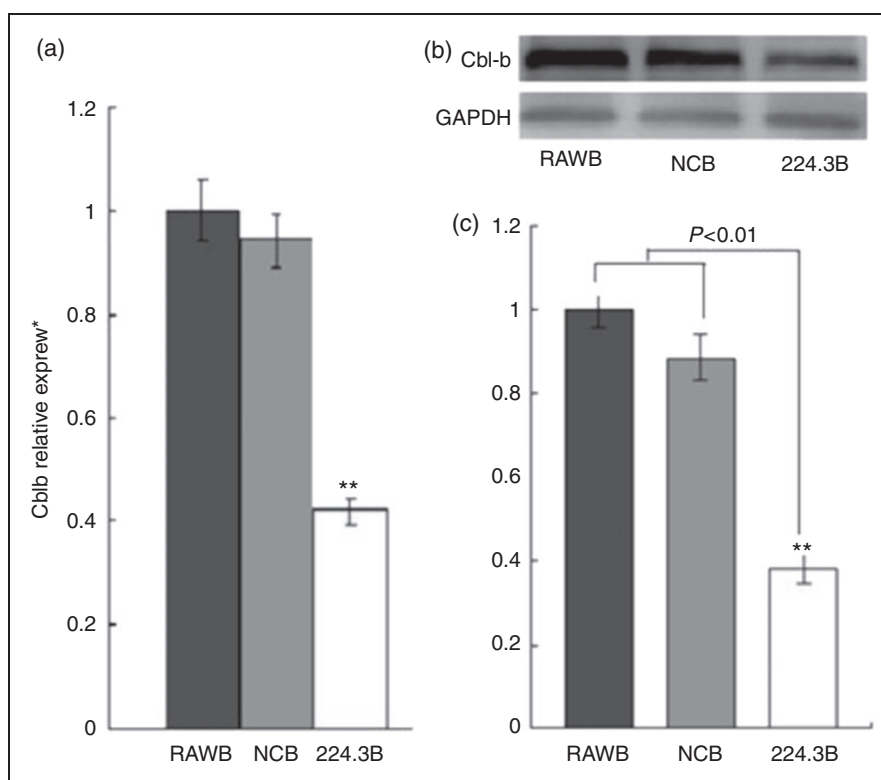
Figure 3. Expression of *Cbl-b* was down-regulated in 224.3 cells that were stimulated by *B. melitensis* infection. (a) qRT-PCR analysis. Lane 1, RAW264.7[+]*B. melitensis*; lane 2, NC[+]*B. melitensis*; lane 3, 224.3[+]*B. melitensis*. (b) Western blot analysis, GAPDH was used as loading control. (c) The quantification of the Western blots for protein expression with Bandscan 5.0 ($n = 3$).

saturated fatty acid-mediated macrophage activation.[23] *In vivo*, Cbl-b limits the dissemination of *Pseudomonas aeruginosa* through interaction with the type III-secreted effector exotoxin T.[24] In our study, in the mCD14 knock-down RAW264.7 cells challenged with *B. melitensis* M5-90, the Cbl-b expression was down-regulated, which suggested that Cbl-b might be implicated in the interaction between *Brucella* and target cells.

In summary, our present study determined the differential expression of miRNA profiles in CD14-silenced RAW264.7 cells stimulated by *B. melitensis* infection, where mmu-miR-199a-3p and mmu-miR-183-5p were found to be up-regulated. Bioinformatics analysis revealed that the potential targets, *Cbl-b*, was altered; this was confirmed by qRT-PCR and Western blot analysis. These findings suggest that CD14 gene silencing alters specific miRNAs which are directly involved in inflammation of macrophages after *B. melitensis* infection. However, further studies to elucidate the exact function of mmu-miR-199a-3p, mmu-miR-183-5p and Cbl-b are warranted.

Declaration of Conflicting Interests

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