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Original Article

Transcriptome analysis of infected human macrophages between strains of *Brucella melitensis* and an *omp31* mutant

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Abstract

Brucella spp. are small aerobes non-motile Gram-negative coccobacilli that act as facultative intracellular pathogens responsible for zoonotic infections. B. melitensis can survive and replicate within host macrophages, the molecular phenomena of this host: pathogen interaction remain totally unknown. The aim of this work was to evaluate the differences in the response between human macrophages infected with different B. melitensis strains. Comparison of transcriptome data was carried out for identifying differentially expressed genes among different strain infection. We evaluated the THP-1 macrophage molecular response at early stages of infection to different strains of Brucella melitensis (B. melitensis wild- type 133 (BM133), B. melitensis ATCC 23456 (BM16M) and a B. melitensis 133 omp31 mutant (LVM31)). Our analysis revealed intriguing differences in the host cell response to two virulent strains (BM16M and BM133), infection with BM16M led to an over-expression of anti-inflammatory pathways, such as cAMP signaling and PI3K-Akt pathway, and down regulation of inflammatory pathways involving IL1A and IL10 compared to BM133. Mutant strain BMLVM31 induced an activation of the apoptotic process and the absence of Omp31, impaired the inhibition of CASP1 and CASP9 expression. Additionally, the mutation of BMLV31 impairs the evasion of cathepsin D in early stages of the infection. These findings shed light on the intricate molecular interactions between B. melitensis strains and human macrophages, providing valuable insights for understanding the pathogenesis of brucellosis.

Keywords: *Brucella melitensis*, Macrophage, Microarray, Omp31, Transcriptome analysis.

1. Introduction

Brucellosis is a global zoonotic disease that causes chronic and debilitating illness in humans, along with economic losses and abortion in animals. This disease is caused by members of the *Brucella* genus, which are small aerobes, non-motile, Gram-negative, short bacilli and facultative intracellular pathogens [1, 2]. Among these species, *B. melitensis* is the most commonly isolated and virulent in humans and the etiological agent of goat brucellosis [3]. Human brucellosis occurs when contaminated animal products, such as unpasteurized dairy products, are ingested. The pathogenicity of *Brucella* lies in the ability to invade, survive and multiply within phagocytes, like macrophages and dendritic cells. Once phagocytosed, *Brucella* resides within a membrane-bound vacuole known as the *Brucella*-containing vacuole (BCV). This vacuole interacts with early endocytic compartments, preventing fusion with lysosomes and eventually mature into endoplasmic reticulum derived replicative organelles [2, 4, 5].

The molecular mechanisms underlying this host-pathogen interaction are not entirely understood. Therefore, it is crucial to comprehend the molecular mechanisms of *Brucella* intracellular survival and proliferation during infection to prevent brucellosis and develop vaccines. To elucidate these mechanisms, researchers typically infect mouse macrophages and human epithelial cells with different strains of *B. melitensis*, including *B. melitensis* 16M ATCC 23456, wild-type strains, and mutants derived from virulent strains.

Studies have shown that *Brucella* can modify proteins to control intracellular traffic, facilitating successful invasion and replication [6]. Our research group has primarily focused on molecular mechanisms of the host to *B. melitensis* 133 wild type strain infection at early stages. Particularly the expression of SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. For instance, Castañeda et al. evaluated the dynamic expression of VAMP3 (Vesicle-associated mem-

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brane protein 3) in murine macrophages stimulated with *B. melitensis* LPS or the complete bacteria. They observed a temporary increase in VAMP3 expression in response to both stimulations, but silencing VAMP3 did not significantly affect *Brucella* intracellular survival [7]. On the other hand, the silencing of STX4 (syntaxin 4) expression led to reduced survival at 2 and 12 hours post-infection, despite no significant differences in its expression during infection [6].

B. melitensis LVM31 attenuated strain, derived from the virulent *B. melitensis* 133 wild-type strain by inserting a kanamycin cassette into *omp31*. This mutant exhibits an alteration of the outer membrane properties, a significant decrease in the internalization, survival and replication in murine macrophages J774.A1 and HeLa cells. It also exhibited a reduced splenic colonization in mice and provided similar protection as the *B. melitensis* Rev1 vaccine in mice [8, 9]. However, a detailed molecular response from the host to this mutant strain remains to be explored.

Due to the need to better understand the functions of human macrophages in innate and adaptative immune responses in brucellosis, we employed microarray technology to reveal the dynamic changes in gene expression at early infection times (2 and 4 hours post-infection) with different *B. melitensis* strains. Our research aims to identify key genes involved in the host response, which can guide future investigations into their roles and effects during infections. Human cDNA microarrays were used to compare early gene expression differences in response to the infection with different *B. melitensis* strains (BM16M, BM133 and BMLVM31).

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Saturated cultured of a frozen glycerol stock of *Brucella melitensis* 16M ATCC 23456 (BM16M) (smooth and virulent), wild-type *B. melitensis* 133 (BM133) (smooth and virulent) and the mutant *B. melitensis omp31::*Kan (BMLVM31) (smooth and attenuated) were cultured each into *Brucella* broth (BB; Difco Laboratories) and incubated with shaking (150 rpm) at 37°C with 5% CO_2 for 24h. Then, a 1/50 dilution was made and subcultured under the same growth conditions, until the late-log growth phase was reached [10]. Growth curves of cultures were determined by counting bacterial colony forming units (CFU), bacterial numbers were assessed by plating a serial dilution on *Brucella* agar (BB, Difco Laboratories) and incubating at 37°C with 5% CO_2 for 72h. All experiments with *B. melitensis* strains were performed in a Biosafety Level 3 facility.

2.2. Mammalian cell culture

Human monocytes THP-1 cells (ATCC TIB-202) were grown in RPMI-1640 (Gibco-Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen), 25 mM HEPES (USB-Affymetrix), 2 mM L-glutamine (Gibco-Invitrogen), 1 mM sodium pyruvate (Gibco-Invitrogen), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich) and a mixture of 100 U/ml of penicillin and 100 µg/ml of streptomycin (Gibco-Invitrogen) at 37°C and 5% CO_2. For infection, 3.5 x 10^6 cells per well were cultured in 6-well plates and subsequently differentiated into macrophage cells by stimulation with 25 nM phorbol-12-myristate-13 acetate (PMA; Sigma) per well for 48 h, washed with RPMI 1640 medium and incubated for 24 h with supplemented media [11].

2.3. Infection and determination of invasiveness

Macrophages were cultured as previously indicated and infected at a MOI of 100:1 with RPMI media supplemented with 10% SFB (Gibco-Invitrogen). The plates were centrifuged at 500 rpm for 10 min to synchronize phagocytosis and then incubated at 37°C in a 5% CO_2 atmosphere for 1 h. Time zero was set at this point. Then cells were washed with media and incubated with media containing 100 ug/ml of gentamicin (Gibco-Invitrogen) to kill extracellular bacteria for 1 h. After antibiotic treatment, cultures were washed 3 times with PBS and re-incubated in fresh medium for 2 and 4 h.

To determine the viable number of intracellular bacteria, cultures were washed with PBS and then lysed with 0.1% Triton X-100 (Sigma) for 10 min. Lysates were serially diluted and culture on *Brucella* agar plates for quantification of CFU. All infections and quantifications were repeated independently at least 3 times.

2.4. RNA extraction, preparation and slide hybridization

After incubating with the bacteria for 2 and 4 h, total RNA was isolated by phenol extraction with TRIzol (Invitrogen), using the standard TRIzol reagent protocol.

Printing of arrays. Human 50-mer oligo library set "A" from MWGBiotech Oligo Sets (http:/www.mwgbiotech.com) was used. It contains 10,000 gene-specific oligonucleotide probes representing the best-annotated genes from human. Oligo library was resuspended to 50µM in Micro Spotting solution (ArrayIt Brand Products). SuperEpoxy coated slides 25x75 mm (ArrayIt Brand Products) were printed in duplicate and fixed at 80°C for 4 hours. For prehybridization the slides were re-hydrated with water vapor at 60°C and fixed with two cycles of UV light (1200J). After boiling for two minutes at 92°C, slides were washed with 95% ethanol for one minute and prehybridzed in 5X SSC, 0.1% SDS and 1% BSA for one hour at 42°C. The slides were washed and dried for further hybridization.

Probe preparation and hybridization to arrays. 10 µg

Table 1. Experimental design of microarray slides and samples.

Experiment	Microarray	Sample- Alexa$_{555}$	Sample-Alexa$_{647}$
A	H10K_07_39	2 h p.i. with BM133	2 h p.i. with BM16M
B	H10K_07_40	2 h p.i. with BM133	2 h p.i. with BMLVM31
C	H10K_07_41	2 h p.i. with BM16M	2 h p.i. with BMLVM31
D	H10K_07_42	4 h p.i. with BM133	4 h p.i. with BM16M
E	H10K_07_43	4 h p.i. with BM133	4 h p.i. with BMLVM31
F	H10K_07_44	4 h p.i. with BM16M	4 h p.i. with BMLVM31

of total RNA was used for cDNA synthesis incorporating dUTP-Alexa555 or dUTP-Alexa647 employing the Firs-Strand cDNA labeling kit (Invitrogen). Incorporation of fluorophore was analyzed by using the absorbance at 555 nm for Alexa555 and 650 nm for Alexa647. Equal quantities of labeled cDNA were hybridized using hybridization solution UniHyb (TeleChem International INC) (Table 1). The arrays were incubated for 14 h at 42°C, and then washed tree times with 1X SCC, 0.05 % SDS at room temperature.

2.5. Data analysis.

Microarray data analysis was performed with free software genArise, developed in the Computing Unit of Cellular Physiology Institute of UNAM (http://www.ifc.unam.mx/genarise/). GenArise carries out a number of transformations: background correction, lowess normalization, intensity filter, replicates analysis and selecting differentially expressed genes. The goal of GenArise is to identify which of the genes show good evidence of being differentially expressed. The software identifies differentially expressed genes by calculating an intensity-dependent z-score. Using a sliding window algorithm to calculate the mean and standard deviation within a window surrounding each data point and define a z-score where z measures the number of standard deviations a data point is from the mean.

$zi = (Ri - mean(R)) / sd(R)$

Where zi is the z-score for each element, Ri is the log-ratio for each element, and sd(R) is the standard deviation of the log-ratio. With this criterion, the elements with a z-score > 2 standard deviations would be the significantly differentially expressed genes.

Statistical analysis of the CFU values, was performed to compare multiple groups by one-way ANOVA. Differences were considered statistically significant at a p-value <0.05.

3. Results

3.1. Invasion and intracellular survival of *B. melitensis* in human macrophages

The difference in cellular internalization and intracellular survival was analyzed at 2 and 4 h (Fig 1). A significant decrease (P<0.05) in internalization and intracellular survival of the mutant strain BMLVM31 was observed at 2 and 4 h as compared to the wild-type strains BM133 and BM16M (Fig 1). Altogether, these results indicate that the mutant BMLVM31 attaches and internalizes less efficiently compared to the parental strain BM133 and BM16M.

3.2. Differential expression between infected human macrophages at 2 h p.i.

BM16M infection-induced alteration in the signal intensity values of 451 different genes (291 up- and 160 down-regulated) in macrophages at 2 h p.i compared to infection with BM133 at 2 h p.i. (Experiment A). BMLVM31 infection-induced alteration in the signal intensity values of 464 different genes (314 up- and 150 down-regulated) in macrophages at 2 h p.i compared to infection with BM133 at 2 h p.i. (Experiment B). BMLVM31 infection-induced alteration in the signal intensity values of 473 different genes (249 up- and 232 down-regulated) in macrophages at 2 h p.i compared to infection with BM16M at 2 h p.i. (Experiment C).

3.3. Differential expression between infected human macrophages at 4 h p.i.

BM16M infection-induced alteration in the signal intensity values of 462 different genes (229 up- and 233 down-regulated) in macrophages at 4 h p.i compared to infection with BM133 at 4 h p.i. (Experiment D). BMLVM31 infection-induced alteration in the signal intensity values of 501 different genes (307 up- and 194 down-regulated) in macrophages at 4 h p.i compared to infection with BM133 at 4 h p.i. (Experiment E). BMLVM31 infection-induced alteration in the signal intensity values of 455 different genes (236 up- and 219 down-regulated) in macrophages at 4 h p.i compared to infection with BM133 at 4 h p.i. (Experiment F)

3.4. Pathway analysis

To understand more specific cellular responses through activated or inactivated pathways and networks utilized by the genes, pathways were mapped using the KEEG database (Tables 2 and 3).

3.5. Gene Ontology category

The gene ontology (GO) term has three ontologies, including biological process, molecular function and cellular component. In this work, only one ontology was evaluated: Biological process. The genes that changed significantly in experiment A were involved in transcription, negative regulation of apoptotic process, regulation of cell proliferation, MAPK cascade, inflammatory response and type I interferon signaling pathway. In experiment B, the genes were involved in regulation of transcription, regulation of cell proliferation, negative regulation of apoptotic process, leukocyte migration, protein transport and type I interferon signaling pathway. In experiment C, the genes were involved in regulation of transcription, negative regulation of apoptotic process, negative regulation of cell proliferation, protein phosphorylation, viral process, cell adhesion, cell-cell signaling, vesicle-mediated transport, cytokine-mediated signaling pathway, type I interferon signaling pathway and transmembrane transport. In experiment D, the genes were involved in negative regulation of cell proliferation, viral process, transcription, apoptotic process, metabolic process, adaptive immune response and exocytosis. In experiment E, the genes were involved

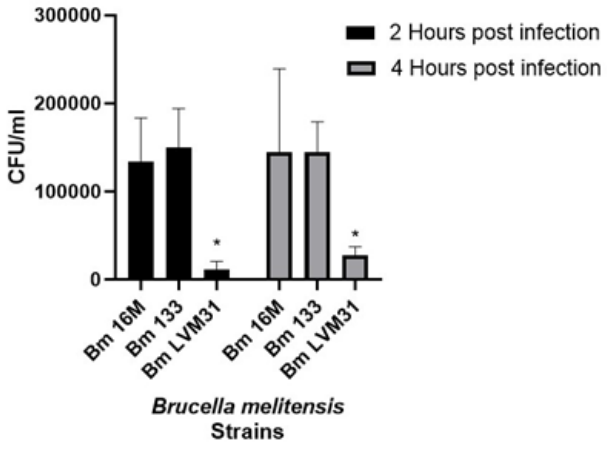

Fig. 1. Intracellular survival of *B. melitensis* strains in the THP-1 macrophage cells. Intracellular growth of *B. melitensis* 16M, 133 and mutant LVM31. The experiment was repeated three times and data represent mean ±SD.

Table 2. KEEG Pathways in *B. melitensis* infected macrophages at 2 h p.i.

Pathways in experiment A (BM16M infection compared to BM133 infection 2 h p.i.)		
Up-regulated pathway	**Genes**	**P-value**
cAMP signaling pathway	*ADCY6, ADRB1, GIPR, PPARA, PDE4B, RAC2, RRAS2.*	7,3E-2
PPAR signaling pathway	*APOA1, DB1, PPARA, UCP1.*	7,7E-2
Down-regulated pathway	**Genes**	**P-value**
PI3K-Akt signaling pathway	*CHAD, FGF5, FGFR2, IFN8, LPAR6, RAC1, SPP1, SKT11, YWHAZ.*	1,3E-2
MAPK signaling pathway	*IL1A, RAC1, CACNG5, DUSP3, DUSP5, FGF5, FGFR2.*	2,8E-2
Herpes simplex infection	*IFNA8, IFNGR2, TAF9B, IFIT1, NXF5.*	8,6E-2
Leishmaniasis	*IFNGR2, ILA1, IL10, PTGS2*	2,7E-2
Pathways in experiment B (BMLVM31 infection compared to BM133 infection 2 h p.i.)		
Up-regulated pathway	**Genes**	**P-value**
cAMP signaling pathway	*ROCK1, ADRB1, CNGB1, PDE4B, PDE4C, RAC2, RRAS2.*	4,7E-2
Leukocyte transendothelial migration	*ROCK1, CYBA, ITGAL, MAPK12, MYL2, RAC2.*	1,8E-2
Osteoclast differentiation	*FOSL2, FCGR3B, CYBA, LILRB3, MAPK12, SIRPG.*	2,9E-2
Sphingolipid signaling pathway	*ROCK1, CTSD, MAPK12, RAC2, S1PR4.*	7,4E-2
Down-regulated pathway	**Genes**	**P-value**
PI3K-Akt signaling pathway	*CHAD, FGFR2, GYS1, IFNA8, LPAR6, RAC1, SPP1, SKT11, YWHAZ.*	6,4E-3
Pathways in cancer	*TFG, DPAK1, EDNRB, FGFR2, LPAR6, RAC1, TGFB2.*	9,8E-2
Choline metabolism in cancer	*RAC1, DGKA, SLC22A3, SLC22A5*.	4,9E-2
Pathways in experiment C (BMLVM31 infection compared to BM16M infection 2 h p.i.)		
Down-regulated pathway	**Genes**	**P-value**
MAPK signaling pathway	*RAC1, IL1A, TGFB2, TAOK3, DUSP3, DUSP5, FGFR2, NRAS, NTF4.*	1,1E-2
Axon guidance	*RAC1, NCK1, EFNB3, NRAS, SEMA3C.*	6,7E-2
Hepatitis B	*IFN8, TGFB2, NRAS, DDX3X, YWHAZ.*	9,7E-2

Table 3. KEEG Pathways in *B. melitensis* infected macrophages at 4 h p.i..

Pathways in experiment D (BM16M infection compared to BM133 infection 4 h p.i.)		
Up-regulated pathway	**Genes**	**P-value**
MAPK signaling pathway	*RAP1B, RAPGEF2, TOAK3, CACNA1C, CACNA1G, DAXX, SRF.*	6,0E-2
Viral carcinogenesis	*MAD1L1, CDKN2B, EGR2, IL6ST, SRF, YWHAB.*	7,6E-2
Down-regulated pathway	**Genes**	**P-value**
Ras signaling pathway	*RAC1, FGFR2, RASA3, HTR7, RALA, PIK3R3.*	4,6E-2
Osteoclast differentiation	*RAC1, IL1A, CALCR, CYLD, LILRA1, LILRA2, PIK3R3.*	1,2E-2
Influenza A	*IL1A, TLR7, IFNA8, HLA-DMA, PIK3R3, RAE1, PRKCB.*	4,1E-2
Pancreatic secretion	*RAC1, BST1, RAB3D, PLA2G5, PRKCB.*	4,6E-2
VEGF signaling pathway	*RAC1, PIK3R3, PTGS2, PRKCB.*	5,9E-2
Fc Epsilon RI signaling pathway	*RAC1, PRKCB, PIK3R3, CSF2.*	7,6E-2
Pathways in experiment E (BMLVM31 infection compared to BM133 infection 4 h p.i.)		
Up-regulated pathway	**Genes**	**P-value**
Legionellosis	*CASP1, CASP9, CXCL8, C3, HSPA2.*	2,3E-2
Amyotrophic lateral sclerosis (ALS)	*CASP1, CASP9, DAXX, SLC1A2*	8,0E-2
Influenza A	*CASP1, CASP9, CXCL8, DNAJB1, FDPSD, HSPA2, TNFSF10, HLA-DQA1, HNRNPUL1.*	2,4E-2
Down-regulated pathway	**Genes**	**P-value**
PI3K-Akt signaling pathway	*RAC1, IFNA8, CHAD, FGFR2, PIK3R3, PPP2R3C, STK11, YWHAZ.*	9,0E-3
ABC transporters	*ABCB7, ABCB9, ABCC1, ABCG4.*	1,3E-2
PPAR signaling pathway	*ACSL4, FABP5, FADS2, SORBS1.*	3,9E-2
Drug metabolism-cytochrome P450	*UGT1A3, UGT2B28, ADH6, GSTM4.*	4,0E-2
Metabolism of xenobiotics by cytochrome P450	*UGT1A3, UGT2B28, ADH6, GSTM4.*	5,0E-2
Chemical carcinogenesis	*UGT1A3, UGT2B28, ADH6, GSTM4.*	6,0E-2
Pathways in experiment F (BMLVM31 infection compared to BM16M infection 4 h p.i.)		
Down-regulated pathway	Genes	P-value
Rap1 signaling pathway	*RAC1, RALA, ADORA2B, CTNNB1, EFNA2, FGFR1, FGFR2, FLT1.*	1,9E-2
PPAR signaling pathway	*ACSL4, APOC3, FABP4, FABP5.*	5,5E-2
Adherens junction	*RAC1, FGFR1, CTNNB1, SSX2IP*	6,4E-2

in signal transduction, regulation of apoptotic process, transcription, proteolysis, inflammatory response, immune response, exocytosis and transmembrane transport. In experiment F, the genes were involved in cell adhesion, transcription, oxidation-reduction process, cell adhesion, apoptotic process, cell proliferation, MAPK cascade and lysosome organization (Fig. 2 and 3)

4. Discussion

B. melitensis is an intracellular pathogen that can survive and replicate inside professional phagocytic cells and it can lead to chronic infection in humans, since the bacteria modulate the host immune response. But the molecular mechanisms involved in the infection have not been well characterized. Previous studies have used human epithelial cells or murine cells (macrophage and epithelial cells), but only a few have characterized the molecular response in human macrophages [12, 13, 14, 15].

Our initial results indicate that *B. melitensis* LVM31 internalize less efficiently than *B. melitensis* 133 and 16M. This result is in line with those obtained by Verdiguel et al., who generated this mutant and found that the intracellular survival of LVM31 was lower in human epithelial cells (HeLa cells) and murine macrophages (J77.4A1 cells) compared to intracellular survival of the parental strain BM133. Also, Zhang *et al*. demonstrated that the mutation of *omp31* of *B. melitensis* 16M, impaired the ability of the bacteria to replicate in murine macrophages (RAW264.7) (16). Even though, all strains were in the same growth conditions, as described by Rosseti *et al.* the late-log growth phase is the most invasive culture compared to the stationary growth phase (least invasive culture), this can indicate that this mutation attenuates the invasiveness of *B. melitensis* [8, 10,16].

In this work, microarray was applied to investigate the differentially expressed genes between two infections. The study was targeted to gain insight into the differences between the response to different strains of *B. melitensis* in human macrophages at 2 and 4 h p.i.

Analysis of the KEEG pathways in experiment A (response to BM16M compared to response to BM133 2 h p.i.), revealed that molecular response to BM16M infection, had an anti-inflammatory profile because cAMP signaling pathway was up-regulated. Erdogan *et al.* demonstrated that expression of cAMP, down-regulates TNFα expression and up-regulates IL-10 expression [17]. This group in 2008, showed that *B. melitensis* infections up regulates cAMP and this, had a suppressing effect on IL-12 expression. On the other hand, PI3K-Akt signaling was down regulated, PI3K-Akt (Phosphatidylinositol 3-kinase) promotes binding of Toll-IL-1 receptor (TIR)-containing proteins on the plasma membrane and TLR signaling. *Brucella* prevents this by expressing TIR-containing proteins and evade the proinflammatory response [2]. *IL1A* (Interleukin 1 alpha) and *IL10* (Interleukin 10) are down regulated; IL1α is a powerful regulator of the immune response. *B. abortus* infection stimulates the expression of this cytokine; Hop et al. showed that interference in IL1α expression, notably augmented susceptibility of murine macrophages to *Brucella* infection, indicating that IL1α is required for efficient clearance of the bacteria [18, 19]. Interleukin 10 is an anti-inflammatory cytokine, expression of IL-10 helps *Brucella* to evade phagolysosome fusion. *IL10* down-regulation can be explained by *IL1A* down-regulation, because blocking IL-1α expression reduced induction of IL-10, IL-1β and TNF [18, 19]. These results, suggest that BM16M can evade immune response better than BM133. Other genes in down-regulated pathways are involved in immune response, like *IFN8* (Interferon alpha 8), *IFNGR2* (Interferon gamma receptor 2), *IFIT1* (Interferon-induced protein with tetratricopeptide repeats 1) but there is no direct evidence with *Brucella* infections.

In experiment D (response to BM16M compared to response to BM133 4 h p.i.) an up-regulation of MAPK signaling pathway was observed, *Brucella* infection stimulates MAPK pathway activation in epithelial cells. Blocking MAPK expression, results in decreased internalization and impaired intracellular replication of *B. melitensis* in HeLa cells [20]. The opposite of what Jimenez et al. observed in murine macrophage, where *Brucella* infection does not up-regulate MAPK pathway [21]. In another study, it was demonstrated that *B. melitensis* activates this pathway in human monocytes and it is necessary for intracellular replication [22]. This down-regulation suggests, that BM16M do not stimulate this pathway the same way that BM133 does. Also, at 4 h p.i., *IL1A* and *TLR7* were down-regulated. *TLR7* codes a Toll-Like Receptor 7, receptor that detects single-stranded RNA. Campos et al. stimulated dendritic cells with *B. abortus* RNA and observed IL-12 and IL-6 secretion [23], this suggest that the

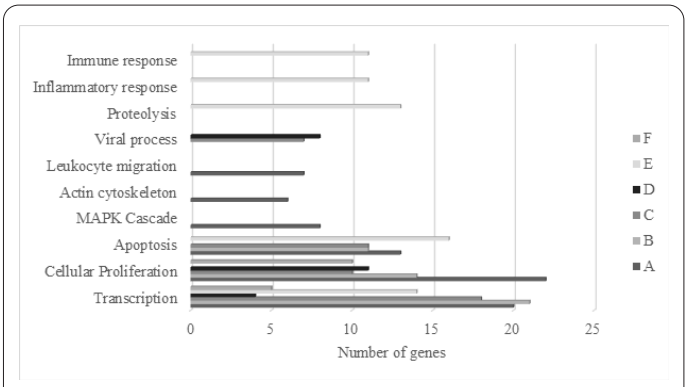

Fig. 2. Categorization by biological processes of differentially up-regulated expressed genes during infections. Up-regulated biological process. A: BM16M vs BM133 infection at 2 h p.i. B: BM16M vs LVM31 at 2 h p.i. C: BM133 vs BMLVM31 infection at 2 h p.i. D: BM16M vs BM133 infection at 4 h p.i. E: BM16M vs LVM31 at 4 h p.i. F: BM133 vs BMLVM31 infection at 4 h p.i.

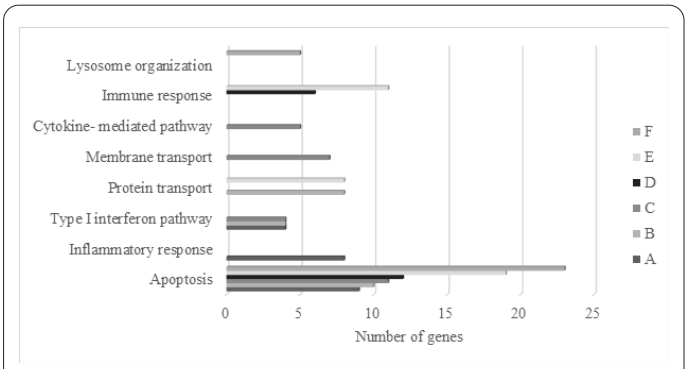

Fig. 3. Categorization by biological processes of differentially down-regulated expressed genes during infections. Down-regulated biological process. A: BM16M vs BM133 infection at 2 h p.i. B: BM16M vs LVM31 at 2 h p.i. C: BM133 vs BMLVM31 infection at 2 h p.i. D: BM16M vs BM133 infection at 4 h p.i. E: BM16M vs LVM31 at 4 h p.i. F: BM133 vs BMLVM31 infection at 4 h p.i.

complete bacteria could stimulate TLR7 expression, just like we observed in this work.

Experiment B, (response to BMLVM31 compared to response to BM133 2 h p.i.), revealed that molecular response to BMLVM31 infection had an up-regulation of *CSTD,* that codes a lysosomal hydrolase Cathepsin D. Suggesting that, the low number of LVM31 CFU at 2 h p.i. maybe due to the presence of cathepsin D, which indicates phagosome fusion with lysosomes. Some studies indicate that *Brucella*-containing vacuole (BCV) do not acquire cathepsin D, because virulent strains inhibit fusion of the phagosome with lysosomal compartments, compared to infections with mutant or attenuated strains, that BCVs are positive to cathepsin D and cannot avoid fusion with lysosomes [24, 25, 12, 26, 27]. This infection also results a down-regulation of *RAC1* (Ras-related C3 botulinum toxin substrate 1 rho family, small GTP binding protein Rac1), which codes Rac1 protein, a master regulator of lamellipodium formation and modulates actin cytoskeleton. Some studies with *Brucella* infections, indicate that virulent strains stimulate Rac1 expression compared to mutant strains [28, 29], as shown in Fig 1. the mutant invades less, so this can be related to less *RAC1* expression (Fig 1.)

At 4 h p.i. (Experiment E), an up-regulation of *CASP1* (Caspase 1) and *CASP9* (Caspase 9) was observed. Caspase 1 activates inflammatory response (IL-1β, IL-18) and pyroptosis and Caspase 9 is an activator of apoptosis process [30, 16, 31]. Zhang et al. in 2016, demonstrated that OMP31 of *Brucella* plays an important role during infection because it inhibits the apoptosis of host cells to benefit intracellular survival and replication, infections with a mutant in *omp31*, activate the apoptotic process and inflammasome, lower intracellular survival rate, higher expression of TNF-α, caspase 8, caspase 3, caspase 9 and cytochrome c [16]. Experiment F, (response to BMLVM31 compared to response to BM16M 4 h p.i.), revealed that molecular response to BMLVM31 infection had a down regulation of *RAC1*. As previously described, this maybe due to a membrane modification of the bacteria and an impaired invasion (Fig 1).

5. Conclusion

In summary, we analyzed the transcriptional differences of different strains of *B. melitensis* during early infection time points in human macrophage cells. We conclude that there is a different response of the host cell to two virulent strains (BM16M and BM133), infection with BM16M stimulates over-expression of anti-inflammatory pathways compared to BM133, where *IL1A* and *IL10* were differentially expressed. Mutant strain BMLVM31 activates the apoptotic process, and the absence of OMP31, impaired the inhibition of *CASP1* and *CASP9* expression.

Further studies are required to confirm these accusations, we will perform qPCR, gene expression silencing and protein analysis to assess this.

Conflicts of interest

The authors declare that they have no competing interests.

Consent for publications

The authors read and approved the final manuscript for publication.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available at the GEO platform with the accession number GSE261048.

Authors contributions

CQ, JR, JM, JP and AV conceived and designed the research. CQ conducted the experiments. JM and LV conducted partial experiments. CQ, JM and JR collected data. CQ, LV, JM and JP analyzed data. CQ, LV, AV wrote the manuscript. All authors red and approved the manuscript.

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