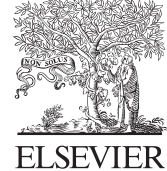
Osong Public Health Res Perspect 2013 4(6), 301—307
http://dx.doi.org/10.1016/j.phrp.2013.10.002
pISSN 2210-9099 eISSN 2233-6052



ORIGINAL ARTICLE

Lon Mutant of *Brucella abortus* Induces Tumor Necrosis Factor-Alpha in Murine J774.A1 Macrophage

Sungdo Park, Young-Sill Choi, Sang-Hee Park, Young-Rok Kim, Hyuk Chu, Kyu-Jam Hwang, Mi-Yeoun Park*

Division of Zoonoses, Korea National Institute of Health, Osong, Korea.

Received: September 9, 2013
Revised: September 30, 2013
Accepted: October 2, 2013

KEYWORDS:
Brucella abortus,
lon mutant,
immune response

Abstract

Objectives: The objective of this study was to isolate a *Brucella lon* mutant and to analyze the cytokine response of *B. lon* mutant during macrophage infection.

Methods: A wild-type *Brucella abortus* strain was mutagenized by Tn5 transposition. From the mouse macrophage J774.A1 cells, total RNA was isolated at 0 hours, 6 hours, 12 hours, and 24 hours after infection with *Brucella*. Using mouse cytokine microarrays, we measured transcriptional levels of the cytokine response, and validated our results with a reverse transcriptase-polymerase chain reaction (RT-PCR) assay to confirm the induction of cytokine messenger RNA (mRNA).

Results: In host J774.A1 macrophages, mRNA levels of T helper 1 (Th1)-type cytokines, including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-2 (IL-2), and IL-3, were significantly higher in the *lon* mutant compared to wild-type *Brucella* and the negative control. TNF-α levels in cell culture media were induced as high as 2 μg/mL after infection with the *lon* mutant, a greater than sixfold change.

Conclusion: In order to understand the role of the lon protein in virulence, we identified and characterized a novel *B. lon* mutant. We compared the immune response it generates to the wild-type *Brucella* response in a mouse macrophage cell line. We demonstrated that the *B. lon* mutants induce TNF-α expression from the host J774.A1 macrophage.

1. Introduction

Brucellosis is an important zoonotic disease affecting many mammalian species, and it can be transmitted to humans [1]. Brucellosis is caused by *Brucella* species, which are small, gram-negative coccobacilli, and this facultative intracellular bacterial pathogen is capable of causing abortion and male infertility in animals. *Brucella abortus* causes abortion in cattle and chronic infections in humans, and symptoms include undulant fever, endocarditis, and arthritis [2]. Prevention of brucellosis in livestock is achieved using a laboratory-derived vaccine

*Corresponding author.
E-mail: miyeoun@korea.kr

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Copyright © 2013 Korea Centers for Disease Control and Prevention. Published by Elsevier Korea LLC. All rights reserved.

strain, which induces the production of antibodies to *Brucella*. There is no available human vaccine [3].

The cell-mediated immune response to brucellosis includes the production of cytokines that activate macrophages for antibrucella activities. However the mechanisms by which these cytokines lead to macrophage activation are complex [4]. Macrophages are particularly important for the survival and spread of *Brucella* during infection. Among the key activities of cytokines, gamma interferon has been shown to induce antimicrobial activities against a variety of intracellular pathogens, and tumor necrosis factor (TNF) can elevate macrophage stimulating factor and increases H_2O_2 production and nitrite release [5].

B. abortus avoids stimulation of the host immune system, permitting the establishment of chronic infection. Some *Brucella* species inhibit host cell apoptosis, similar to *Chlamydia, Bartonella henselae*, and *Mycobacterium bovis*. Generally, *B. abortus* infection induces minimal levels of cytokines [6,7].

Lon protease contributes to the degradation of short-lived, misfolded, or damaged proteins by environmental changes, and seems to play an important role as a chaperone [8]. In *Escherichia coli*, lon is responsible for adenosine triphosphate-dependent proteolysis and is thought to play a major role in the response to environmental stress by downregulating *SulA*, an inhibitor of cell division, and by modulating the activity of the transcriptional activator *RcsA* [9,10]. In previous research, a *lon* mutant of *Brucella* was studied for its response to environmental stress conditions and the role of *lon* in the maintenance of chronic infection was investigated in the mouse model. Although the biochemical functions of *B. abortus* lon mutant and its effect on *Brucella* pathogenesis both *in vivo* and *in vitro* were investigated more than 10 years ago, the induction of TNF-α in macrophages infected by *Brucella lon* mutant has not yet been reported [11]. TNF-α is an important player in the apoptotic pathway and may be involved in directing cell death toward apoptotic or necrotic pathways. Furthermore, TNF-α mRNA expression peaks at early infection times with *E. coli* K12 or heat-killed *Brucella*-infected cells. However, there is no expression of mRNAs encoding TNF-α in live *Brucella* infected cells. For example, *Brucella suis* infected macrophages express interleukin (IL)-1, IL-6, IL-10, IL-8, but do not express TNF-α [12]. The viability of the *B. lon* mutant during infection and its effect on the immune response are largely unknown.

2. Materials and methods

2.1. Bacteria growth conditions

The characteristics of *Brucella* strains were confirmed by using AMOS (*B. abortus-Brucella melitensis-Brucella ovis-B. suis*) polymerase chain reactions (PCRs). The bacterial strains used were *B. abortus* 2308, wild-type strain of *B. abortus* (parent strain of *lon* mutant) and *lon* mutant. We isolated the wild-type strain of *B. abortus* from the blood sample of the patient and seeded in *Brucella* agar plate. Briefly, this wild-type strain of *B. abortus* was isolated from blood cultures of a 33-year-old man with acute brucellosis, who presented with fever [13]. To confirm bacterial species, genomic DNA was isolated from samples of *B. abortus*, wild-type *Brucella* strain and *lon* mutant, and PCR amplification of 16S ribosomal RNA (rRNA) was performed. *Brucella* strains were cultured in tryptic soy broth (TSB; Difco, Detroit, MI, USA) medium at 37 °C in an atmosphere containing 5% CO_2. Kanamycin was added at a concentration of 50 μg/mL unless otherwise indicated. *E. coli* strains were cultured at 37 °C in Luria-Bertani (LB) medium. All work with live *B. abortus* was performed at biosafety level 3. The J774.A1 macrophage cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA). During *Brucella* infection, cell viability was evaluated by trypan blue exclusion assay. Apoptotic and necrotic cells were analyzed by flow cytometry (FAScan, BD Bioscience, San Jose, CA, USA) using Annexin V-FITC/propidium iodide (PI) detection kit (ApoScan, BioBud, Seoul, Korea).

2.2. Construction of *Brucella* mutant library

The mini-Tn5Km2-bearing plasmid pUT mini-Tn5Km2 was introduced into wild-type *B. abortus* isolate from an *E. coli* K-12 derivative, SM17λpir, by conjugation. The pUT vector containing mini-Tn5Km2 was kindly provided by Professor S. Kim, College of Veterinary Medicine, Gyeongsang National University, Korea. The obtained mutants were isolated from *Brucella* agar plates containing kanamycin (30 μg/mL). The chromosomal DNA from mutants was digested with *Eco*RI, cloned to plasmid pBluescript II KS(+), transformed into *E. coli* DH5α, and plated onto LB agar plate containing ampicillin (100 μg/mL) and kanamycin (30 μg/mL). Plasmid DNA from colony was extracted by using the plasmid Mini Kit (Qiagen, Valencia, CA, USA), and the chromosomal DNA sequence was analyzed by using the mini-Tn5Km2 transposon O'-end primer (5'-CCTCTAGAGTCGACCTGCAG-3').

2.3. RNA isolation, reverse transcriptase-polymerase chain reaction, and microarray analysis

Brucella cultures derived from different *Brucella* colonies were used to infect different groups of macrophage cells to obtain independent infections. Following 1.5-hour incubation at 37 °C in 5% CO_2, the cells were washed three times with phosphate buffered saline (PBS). At 0 hours (no *Brucella* infection) and at 30 minutes, 1 hour, and 24 hours postinfection, 7.5 mL of

Table 1. Primers for reverse transcriptase-polymerase chain reaction used in this study

Primer name	Primer sequence (5'-3')
β2-microglobulin-F	GGCTCGCTCGGTGACCCTAGTCTTT
β2-microglobulin-R	TCTGCAGGCGTATGTATCAGTCTCA
TNF-α-F	AGCCCACGTCGTAGCAAACCACCAA
TNF-α-R	ACACCCATTCCCTTCACAGAGCAAT

TNF = tumor necrosis factor.

TRIzol reagent (Invitrogen Corporation Carlsbad, CA, USA) was added to each flask in a group. Uninfected J774.A1 cells were counted to determine the value for 0 hours postinfection. The homogenized samples were incubated for 5 minutes at room temperature. All RNA samples were further purified using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions and resuspended in RNase-free water. Prior to use, the integrity and concentration of each RNA sample was checked using an Agilent 2100 BioAnalyzer and NanoDrop instrument. RNA samples had a total RNA profile exhibiting a 28S band approximately two times more intense than the 18S ribosomal band and an A260/A280 ratio of 1.9. Primers were designed using the Primer software and were purchased from Bioneer (Daejeon, Korea). To confirm microarray data, the same triplicate biological RNA samples were used for reverse transcriptase-polymerase chain reaction (RT-PCR) experiment. To determine if the changes in cell growth and cell density influenced the macrophage gene expression profiles, only DMEM was added to uninfected J774.A1 cell cultures at 0 hours, 6 hours, 12 hours, and 24 hours with 0 hours defined as it was for the microarray experiment. Twenty nanograms of RNA was used for reverse transcription reaction (Superscript reverse transcription kit, Invitrogen). The primer pair for β2 microglobulin was used for internal housekeeping gene control (Table 1). The PCR conditions were as follows: initial denaturation at 95 °C for 3 minutes, followed by 35 cycles at 95 °C for 1 minute, at 55 °C for 1 minute, and at 72 °C for 1 minute. PCR products were separated on 1.2% agarose gel and photographed. Band intensities were quantified using Chemi analysis (Amersham Pharmacia, Piscataway, USA). For test RNAs, synthesis of target complementary DNA (cDNA) probes and hybridization were performed using Agilent's Low RNA Input Linear Amplification kit (Agilent Technology, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, cDNA master mix was prepared and added to the reaction mixer. The hybridized images were scanned using Agilent's DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technology). The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity. Functional annotation of genes was performed according to Gene Ontology Consortium (http://www.geneontology.org/index.shtml) by GeneSpringGX 7.3.

2.4. Infection of J774A.1 murine macrophages and analysis of cytokines secretion

J774.A1 macrophage cells were plated in 24-well plates in DMEM. The J774.A1 cells were then infected with *B. abortus* strains in triplicate wells of a 24-well plate at a multiplicity of infection (MOI) of 50:1. Following 1.5 hours of incubation at 37 °C in atmosphere containing 5% CO_2, the cells were washed three times with phosphate-buffered saline. Infected cells were lysed with distilled water at 0, 6, 12, and 24 hours postinfection. In addition, supernatants from *Brucella* infected J774.A1 were collected and analyzed for IL-2, IL-4, IL-5, IL-10, IL-12(P70), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, and TNF-α by Bio-Plex Cytokine Assay (Mouse Th1/Th2 Panel, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Bacterial infection and intracellular survival assay were done by modified Kim et al's method [14]. Briefly, *B. abortus* strains were inoculated in J774.A macrophage grown on a 96-well plate with DMEM with 10% fetal bovine serum. After 1.5 hours incubation, cells were washed twice with 0.2 μL of sterile PBS and incubated with DMEM containing 10% FBS. At each time point, cells were washed three times with PBS and were lysed with distilled water. The colony formation unit (CFU) was measured by serial dilutions on *Brucella* agar plates.

2.5. Statistical analysis

The nonparametric trend test was used for comparison of replication rate in host cell between the *Brucella lon* mutant, *B. abortus* 2308, and *B. abortus* wild-type strain. The analysis was performed using the SAS version 9.1 software packages (SAS Institute Inc., Cary, NC, USA). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Construction and culture of B. abortus lon mutant

In order to select a *B. abortus lon* mutant from mutant library, primary screening was performed using

genomic DNA digestion. Genomic *Eco*RI fragments containing the Tn5 insertion were isolated and identified by sequencing analysis. The results from the cloning revealed that Tn5 was inserted into a gene whose putative protein product has homology to the lon protein. Sequence of the open reading frame of the *lon* gene confirmed that Tn5 was inserted to its C-terminal region (409th amino acid). We studied the *B. lon* mutant for *in vitro* phenotypes that might differentiate it from the wild-type. Comparisons of the growth characteristics of the *lon* mutant versus wild-type *Brucella* revealed that their exponential growth rates in rich medium and intracellular growth within J774A.1 macrophage cells were the same (Figure 1).

3.2. Cytokine response of macrophage during lon mutant infection

To clarify the macrophage cytokine response during *B. lon* mutant infection, a time course of microarray analysis was performed at 0 hours, 6 hours, 12 hours, and 24 hours postinfection. The mRNAs whose levels changed more than twofold compared with the levels in the controls were considered in the microarray results. The expressed genes in J774A.1 macrophages were calculated as the normalized fold change during *lon* mutant infection. The gene encoding Cxcl10 was the most significantly overexpressed in J774A.1 by *B. lon* mutant postinfection. Upregulated TNF-α was observed after infection with *Brucella* (Table 2). By Kyoto Encyclopedia of Genes and Genomes pathway analysis, we confirmed that genes encoding proteins involved in TNF signaling were more expressed in the cytokine microarray results. The gene encoding TNF-α was significantly upregulated (6.61-fold) by 6 hours, and TNF receptor was also upregulated (7.78-fold) at later infection times. To validate this upregulation we analyzed TNF-α mRNA (446 bp) transcription by RT-PCR. TNF-α expression was detected in the early stage of *lon* mutant infection at 6 hours postinfection. TNF-α transcription was not increased in the control groups (no infection, *B. abortus*, and wild-type *Brucella*) until 12 hours postinfection (Figure 2).

3.3. TNF-α response

To examine the expression of TNF-α protein, the supernatants of uninfected and *Brucella* infected J774.A1 macrophages were collected at different time points, and the concentrations of cytokines released from macrophages were determined. Secretion of TNF-α after 6 hours of cell culture in J774.A1 ranged from 8.5 ng/mL up to very high levels of 1,068 ng/mL after 1 day of infection (Figure 3). In addition, the expression of IL-12b, IL-10, and GM-CSF were detected at only a very low level (1-36 pg/mL) in culture media under all conditions. IL-10 expression was also increased (20 pg/mL) during infection at the 24-hour time point.

3.4. Growth of lon mutant

We wanted to exclude the hypothesis that the effect of *Brucella* proliferation in infected J774.A1 cells is due to a difference in growth rate. We confirmed the doubling time in broth medium and also performed intracellular replication assays in J774A.1 macrophage cells using the *B. lon* mutant, *B. abortus* 2308, and *B. abortus* wild-type strain. J774.A1 cells were infected with *B. lon* mutant, *B. abortus* 2308, or *B. abortus* wild-type strain. After that, numbers of viable CFU were determined at 1 hour, 6 hours, 12 hours, 24 hours, and 48 hours postinfection (data not shown). Viability

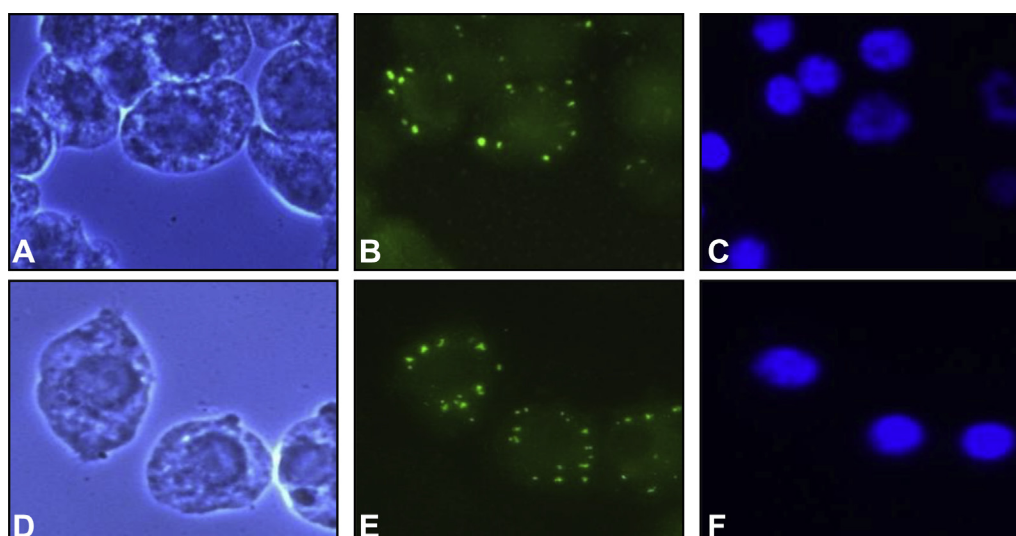


Figure 1. Intracellular localization of *Brucella abortus* wild-type and *lon* mutant in J774.A1 cells. The J774.A1 cells infected with *B. abortus* wild-type (upper panel) or *lon* mutant (lower panel) for 6 hours were fixed and stained with an antibody directed against *Brucella* in the same field. The results were evaluated by bright-field photomicrographs (A and D), fluorescein isothiocyanate (FITC) filter (B and E), and Hoechst staining (C and F) for nuclei. All panels, ×400.

Table 2. Messenger RNA expression of upregulated cytokine genes during *lon* mutant infection between 6 hours and 24 hours postinfection

Normalized fold	Accession number	Gene symbol	Description
6 h			
11.23	NM_021274	Cxcl10	Chemokine ligand 10
6.61	NM_013693	Tnf	Tumor necrosis factor
5.52	NM_008352	IL2b	Interleukin 12b
5.27	NM_009425	Tnfefl0	Tumor necrosis factor superfamily, member 10
4.47	NM_010510	Ifnb1	Interferon beta 1
3.69	NM_011577	Tgfb1	Transforming growth factor, beta 1
12 h			
7.9	NM_021274	Cxcl10	Chemokine ligand 10
7.78	NM_009399	Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a
6.81	NM_013653	Ccl5	Chemokine ligand 5
4.21	NM_010554	Il1a	Interleukin 1 α
3.8	NM_011577	Tgfb1	Transforming growth factor, beta 1
3.18	NM_009425	Tgfsf10	Tumor necrosis factor superfamily, member 10
3.08	NM_028679	Irak3	Interleukin-1 receptor-associated kinase 3
3.07	AK156231	Ccnt2	Cyclin T2
24 h			
5.44	NM_197889	Ifnz	Interferon zeta
5.16	NM_009425	Tnfsf10	Tumor necrosis factor superfamily, member 10
4.57	NM_013653	Ccl5	Chemokine ligand 5
4.25	NM_008003	Fgf15	Fibroblast growth factor 15
3.68	NM_016673	Cntfr	Ciliary neurotrophic factor receptor
3.54	NM_021274	Cxcl10	Chemokine ligand 10
3.36	NM_011888	Ccl19	Chemokine ligand 19
3.35	NM_011577	Tgfb1	Transforming growth factor, beta 1
3.31	NM_021887	Il21r	Interleukin 21 receptor
3.26	NM_009399	Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a

assays showed that the *lon* mutant gave similar number of CFUs as its parent strain at 6 hours postinfection. In infected J774A.1 cells, the replication of wild-type *Brucella* ($p = 0.0392$) and *lon* mutant ($p = 0.0157$) increased significantly in a time dependent manner, which is consistent with the results obtained with a CFU measurement. The replication of *B. abortus* ($p = 0.0517$) also increased but the increase was not significant statistically.

4. Discussion

Brucella alters the expression of many genes to adapt to harsh conditions in the host cell and to survive against the host immune system. The intracellular survival of *B. abortus* has been documented, and it is incorporated into phagosomes and localized to ER like structures in J774.A1 macrophages [15,16]. The macrophage is a central means of defense against microbial pathogens

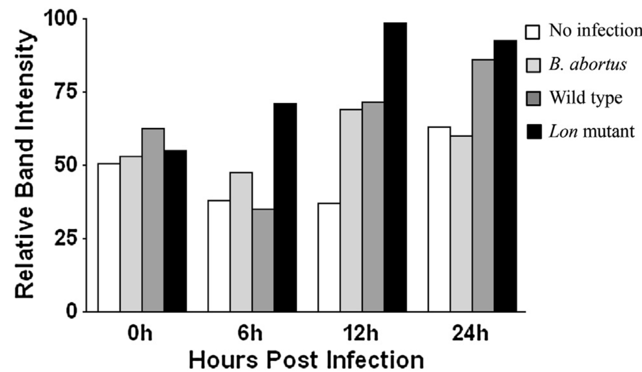


Figure 2. The messenger RNA expression of tumor necrosis factor (TNF)-α during early infection. The level of TNF-α was determined from the J774.A1 cells infected with *Brucella abortus*, wild-type *Brucella,* or the *lon* mutant [multiplicity of infection (MOI) = 50] by reverse transcriptase-polymerase chain reaction.

that kill microorganisms by ingestion. Based on previous studies, it is clear that macrophages play a major role in killing *Brucella*. In acute infection of *B. abortus*, the early immune response is critical to controlling infection. For examples, the activation of neutrophil was accomplished in 60 minutes and early neutrophil activation is followed by IL-8 induction. Mature neutrophil survival and apoptosis are influenced by the inflammatory cytokines [17].

In this study, the transcription and expression of TNF-α was analyzed in the J774.A1 cell line using both RT-PCR on extracted RNA and cytokine assays on supernatant culture media. A significant increase in TNF-α was detected at many time points. The transcript level for TNF-α was dramatically elevated as early as 6 hours after infection with the *B. lon* mutant in this study. The *B. lon* mutant may be involved in TNF-α mediated cell death toward an apoptotic or necrotic pathway. TNF-α is one of the products of macrophage response directly involved in the killing of *Brucella* and the mechanism regulating the TNF-α production in target cells has not yet been clearly defined. The concentration of TNF-α was measured in infected cell media in the amount of 0.5−30 ng/mL during 1 day postinfection based on previous studies [18,19]. This suggests that apoptosis or macrophage cell death occurs in J774.A1 cells infected with *lon* mutant in this study.

Brucella requires enough time for the preparation of growth avoiding the host immune system and space for the *Brucella* replication to be a successful infection [20,21]. It has been shown that the replication rate of *Brucella* in macrophages decreases during the first 12 hours of infection. This decrease seems to be necessary for intracellular adaptation of *lon* mutant from media culture condition. Recently, other groups have reported similar findings in virulent *Brucella* strain [22,23]. The recovery mechanisms and metabolic pathway proteins to establish intracellular *Brucella* adaptation were elucidated by a comprehensive analysis of its proteomes. Because survival and replication in host cells is important when determining whether mutated gene influences the virulence of *Brucella*, we examined the intracellular replication of *Brucella* strains in J774.A1 macrophage cells. We cannot rule out that the decreased viability of the *lon* mutants is a result of the host macrophages rapidly killing of cells during early infection. The ability

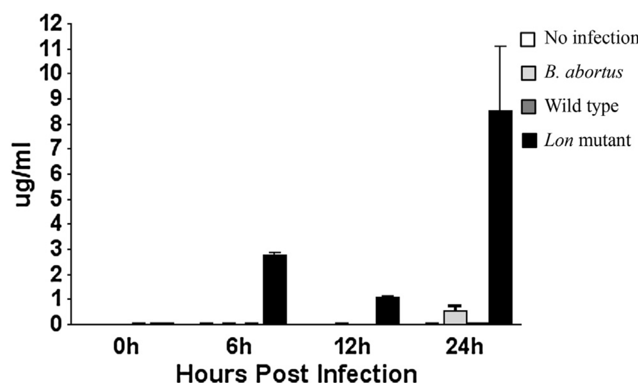


Figure 3. The protein expression of tumor necrosis factor (TNF)-α during early infection. The level of TNF-α was determined from the medium of J774.A1 cells infected with *Brucella abortus*, wild-type *Brucella,* or the *lon* mutant [multiplicity of infection (MOI) = 50] using enzyme-linked immunosorbent assay.

of the *lon* mutant to proliferate is similar to wild-type *B. abortus* and *B. abortus* 2308 in J774A.1 macrophage cells at 24 hours postinfection. By contrast, the majority of *Brucella* cells were killed during the first 24 hours, whereas the surviving bacteria began to replicate 24 hours postinfection. This type of mutant survival pattern is consistent with one report [24].

In *Brucella lon* mutants, our microarray study is the first to show that macrophage cytokine gene expression changes occur at an early infection stage. The most significant finding of this work is the identification of the host cell response during *B. lon* mutant infection and secondly is the correlation between the virulence of the *lon* mutant and macrophage viability. To better understand the role *lon* plays in *Brucella*'*s* ability to evade host immune mechanisms and establish chronic infection, we compared transcriptional profiles of the host cytokine response to infection with wild-type and *lon* mutant *Brucella* strains.

Acknowledgments

This work was supported by the intramural grant of the Korea National Institute of Health (2008-N52001-00 and 2009-N52001-00). We thank Professor Suk Kim at Gyeongsang National University, Korea, for kindly providing mini-Tn5Km2 plasmid. We also extend appreciation to the Division of Bacterial Respiratory Infections, Korea National Institute of Health, for supplying media.

References

1. Ratushna VG, Sturgill DM, Ramamoorthy S, et al. Molecular targets for rapid identification of Brucella spp. BMC Microbiol 2006 Feb;22(6):13−22.
2. Jiang X, Baldwin CL. Effects of cytokines on intracellular growth of Brucella abortus. Infect Immun 1993 Jan;61(1):124−34.
3. Jacob J, Hort GM, Overhoff P, Mielke ME. In vitro and in vivo characterization of smooth small colony variants of *Brucella abortus* S19. Microbes Infect 2006 Feb;8(2):363−71.
4. Pei J, Wu Q, Kahl-McDonagh M, Ficht TA. Cytotoxicity in macrophages infected with rough Brucella mutants is type IV secretion system dependent. Infect Immun 2008 Jan;76(1):30−7.
5. Golding B, Scott DE, Scharf O, et al. Immunity and protection against *Brucella abortus*. Microbes Infect 2001 Jan;3(1):43−8.
6. DeLeo FR. Modulation of phagocyte apoptosis by bacterial pathogens. Apoptosis 2004 Jul;9(4):399−413.
7. Ko J, Splitter GA. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin Microbiol Rev 2003 Jan;16(1):65−78.
8. Huston WM. Bacterial proteases from the intracellular vacuole niche; protease conservation and adaptation for pathogenic advantage. FEMS Immunol Med Microbiol 2010 Jun;59(1):1−10.
9. Kawarai T, Wachi M, Ogino H, et al. SulA-independent filamentation of *Escherichia coli* during growth after release from high hydrostatic pressure treatment. Appl Microbiol Biotechnol 2004 Apr;64(2):255−62.
10. Kuo MS, Chen KP, Wu WF. Regulation of RcsA by the ClpYQ (HslUV) protease in *Escherichia coli*. Microbiology 2004 Feb; 150(2):437−46.
11. Robertson GT, Kovach ME, Allen CA, et al. The *Brucella abortus Lon* functions as a generalized stress response protease and is required for wild-type virulence in BALB/c mice. Mol Microbiol 2000 Feb;35(3):577−88.
12. Billard E, Dornand J, Gross A. Interaction of *Brucella suis* and *Brucella abortus* rough strains with human dendritic cells. Infect Immun 2007 Dec;75(12):5916−23.
13. Ha GY, Choi YS, Kim MY, et al. Diagnostic experience in the 3 human brucellosis cases by the microbiologic, serologic and gene tests. Korean J Clin Micobiol 2007 Oct;10(2):154−9.
14. Kim S, Watarai M, Kondo Y, et al. Isolation and characterization of mini-Tn5Km2 insertion mutants of *Brucella abortus* deficient in internalization and intracellular growth in HeLa cells. Infect Immun 2003 Jun;71(6):3020−7.
15. Lamontagne J, Forest A, Marazzo E, et al. Intracellular adaptation of *Brucella abortus*. J Proteome Res 2009 Feb;8(3):1594−609.
16. Arenas GN, Staskevich AS, Aballay A, Mayorga LS. Intracellular trafficking of *Brucella abortus* in J774 macrophages. Infect Immun 2000 Jul;68(7):4255−63.
17. Dornand J, Gross A, Lafont V, et al. The innate immune response against *Brucella* in humans. Vet Microbiol 2002 Dec;90(1−4): 383−94.
18. Jiménez de Bagüés MP, Terraza A, Gross A, Dornand J. Different responses of macrophages to smooth and rough Brucella spp.: relationship to virulence. Infect Immun 2004 Apr;72(4):2429−33.
19. Rittig MG, Kaufmann A, Robins A, et al. Smooth and rough lipopolysaccharide phenotypes of *Brucella* induce different intracellular trafficking and cytokine/chemokine release in human monocytes. J Leukoc Biol 2003 Dec;74(6):1045−55.
20. He Y, Reichow S, Ramamoorthy S, et al. *Brucella melitensis* triggers time-dependent modulation of apoptosis and down-regulation of mitochondrion-associated gene expression in mouse macrophages. Infect Immun 2006 Sep;74(9): 5035−46.
21. Barquero-Calvo E, Chaves-Olarte E, Weiss DS, et al. *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. PLoS One 2007 Jul; 2(7):e631.
22. Rambow-Larsen AA, Petersen EM, Gourley CR, Splitter GA. B*rucella* regulators: self-control in a hostile environment. Trends Microbiol 2009 Aug;17(8):371−7.
23. Parent MA, Goenka R, Murphy E, et al. *Brucella abortus* bacA mutant induces greater pro-inflammatory cytokines than the wild-type parent strain. Microbes Infect 2007 Jan;9(1):55−62.
24. Weeks JN, Galindo CL, Drake KL, et al. *Brucella melitensis* VjbR and C12-HSL regulons: contributions of the N-dodecanoyl homoserine lactone signaling molecule and LuxR homologue VjbR to gene expression. BMC Microbiol 2010 Jun; 10(8):1−20.