Microbes and Infection 15 (2013) 450–460

www.elsevier.com/locate/micinf

Original article

Replication of *Brucella melitensis* inside primary human monocytes depends on mitogen activated protein kinase signaling

Odyssefs Dimitrakopoulos, Kassiani Liopeta, George Dimitracopoulos, Fotini Paliogianni*

University of Patras Medical School, Department of Microbiology, Patras 26500, Greece

Received 7 November 2012; accepted 18 April 2013
Available online 28 April 2013

Abstract

The clinical course of infections caused by *Brucella* is linked to its capacity to modulate the initial immune response of macrophages in order to ensure its intracellular replication. Signal transduction pathways implicated in the survival of *Brucella* in human cells are not completely elucidated. We herein investigated the involvement of the TLR−MAPK-dependent signaling pathways in the survival of *Brucella* in primary human monocytes using live clinical strains of *Brucella melitensis*. *B. melitensis* caused a delayed, TLR2 dependent MAPK activation. Specific MAPK inhibitors for p38 (SB203580), ERK1/2 (PD98059) and JNK (SP600125) or the anti-TLR2 blocking Ab inhibited both inflammatory and anti-inflammatory responses characterized by TNF-α, IL-6 and IL-10 production. Intracellular replication of *B. melitensis* was mainly dependent on p38 and JNK activation and not affected by IL-10 levels. These are the first evidence to support that survival of *B. melitensis* inside human monocytes depends on interplay among the different MAPK family members, activated through TLR2, in spite of an initial pro-inflammatory response.

Keywords: *Brucella*; MAP kinases; TLR2; Human monocytes

1. Introduction

Brucella, a facultative intracellular bacterium that causes undulant fever, endocarditis, arthritis and osteomyelitis in humans [1], establishes chronic infections by infecting, surviving and replicating in different cell types, including macrophages and dendritic cells [2]. *Brucella abortus*, *Brucella melitensis* and *Brucella suis* are the main species that cause human brucellosis, with *B. melitensis* causing the majority of cases and the most severe symptoms [3].

The capacity of *Brucella* to persist in infected cells depends on its stealthy strategy to avoid or interfere with components of the host innate and acquired immune responses. Initial host defenses against bacterial infection are stimulated by Pathogen Associated Molecular Patterns (PAMP), which are recognized by the host [4]. Toll like receptors (TLRs) are among the first receptors to detect a microbial infection and signal for inflammation [5]. Recognition of PAMP by TLR stimulates the activation of intracellular signaling pathways via Toll/Interleukin-1 receptor (TIR)-domain containing adaptors, including myeloid differentiating factor 88 (MyD88), TIR-domain-containing Adaptor Protein (TIRAP), TIR-domain-containing adaptor-inducing interferon beta (TRIF) and TRIF-related adaptor molecule (TRAM) [6].

Ample evidence implicates the different members of TLR family in recognition of *Brucella* and/or clearance of infection. For instance, TLR2 does not play a role in regulating *B. abortus* infection [7], whereas TLR9 has been shown to be required for clearance of the bacterium in infected mice [8]. On the other hand, signaling through TLR2 stimulates production of pro-inflammatory cytokines by innate immunity cells after stimulation with live *B. abortus*, heat killed bacteria [9] or *Brucella* lipoproteins [10]. TLR4 has been implicated in pro-inflammatory cytokine production triggered by the outer membrane proteins of *B. abortus* [11] or live bacteria [12], but

* Corresponding author. Tel.: +30 2610 999660; fax: +30 2610 994922.
E-mail address: fpal@upatras.gr (F. Paliogianni).

1286-4579/$ - see front matter
http://dx.doi.org/10.1016/j.micinf.2013.04.007

its role in host resistance remains controversial with data supporting [7,13] or arguing against its role [12,14]. Despite the contradictory views about the involvement of TLR2 and TLR4 in resistance against *Brucella*, MyD88 knockout mice are more susceptible to *Brucella* infection, suggesting a crucial role of this signaling adaptor in immune response against the bacterium [8,12,13].

One essential branch of signaling cascades initiated by TLR is the ubiquitously expressed family of mitogen activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3K). MAPK mediate cellular responses to a variety of extracellular stimuli, such as physical stress, inflammatory cytokines, growth factors and bacterial components [15]. MAPK activity has been shown to be critically involved in the pathogenesis of intracellular bacteria such as *Listeria* [16], *Yersinia* spp. [17] and *Mycobacterium* spp. [18]. However, experimental data linking *Brucella* triggered MAPK activation to immune intervention by virulent smooth *Brucella* are limited [19,20]. In this study, we used fresh human monocytes, which detect the bacterium when it enters the bloodstream, to investigate MAPK orchestration of the innate immune response directed against pathogenic live clinical strains of *B. melitensis*. We show for the first time in human cells, that *B. melitensis* causes a delayed but sustained MAPK activation, which is necessary for intracellular replication of the bacterium, and is mediated by TLR2 activation.

2. Materials and methods

2.1. Bacterial culture and stimulation

Clinical strains of *B. melitensis*, originally isolated from the blood of patients with acute brucellosis in the University Hospital of Patras and the vaccine strain Rev.1 defined by (i) resistance to 2–5 μg/ml streptomycin and (ii) inhibition by 5 IU/ml of penicillin [21] were used in our study. Identification at the species level was made according to standard methods including urea hydrolysis, H_2S production, and growth on medium containing basic Fuchsin (1:100,000) or Methionin (1:100,000). Bacteria were grown in Tryptic soy broth (TSB; Sigma–Aldrich) and kept frozen at −80 °C in 15% glycerol TSB medium. For infection 2 ml TSB were inoculated with a single bacterial colony from a freshly streaked TS agar (TSA) plate and grown at 37 °C for 24 h. Twenty-four-hour bacterial cultures (late log phase) were used to infect cells. The bacterial concentration was adjusted to 10^8 organisms/ml, according to the McFarland standards of bioMérieux Inc (Marcy l'Etoile, France). Bacteria were washed and resuspended in RPMI 1640 at the desired concentration immediately before they were used in stimulation experiments with monocytes. Live bacteria were constantly interacting with cells in antibiotics-free media during the experimental procedure.

2.2. Reagents and antibodies

RPMI 1640 cell culture medium containing 10% low-endotoxin fetal bovine serum (FBS) and 40 mg/ml of gentamicin (complete RPMI 1640) and DMEM were purchased from GIBCO® (Invitrogen Carlsbad, CA, USA). *Escherichia coli-lipopolysaccharide* (LPS, 055:B5) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, IL-1β enzyme-linked immunosorbent assay (ELISA) kits, recombinant human IL-10 and monoclonal anti-IL-10, anti-IL-1 and anti-IL-18 neutralizing antibodies (Abs) were purchased from R&D Systems (McKinley Place, MN, USA). Anti-phospho-p38 (Thr180/Tyr182), anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2, Thr202/Tyr204), anti-phospho-c-Jun NH2-terminal kinase (JNK, Thr183/Tyr185) and anti-β-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The p38 inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole (SB203580), the JNK inhibitor Anthra[1,9-*cd*] pyrazol-6(2*H*)-one (SP600125) and the MAPK kinase (MEK) inhibitor 2′-amino-3′-methoxyflavone (PD98059) were obtained from Calbiochem (EMD4Biosciences, Merck KGaA, Darmstadt, Germany). Azide-free monoclonal anti-TLR2 (TL2.1) and anti-TLR4 (HTA125) were obtained from Abcam (Cambridge, UK) and were specified for neutralization experiments. Anti-IgG2a isotype control Ab was also obtained from Abcam. The synthetic bacterial lipopeptide Pam3CSK4 (TLR2 activator) was obtained form Invivogen (San Diego, CA, USA).

2.3. Human cell culture conditions

Peripheral blood mononuclear cells were isolated from freshly collected leukocyte-rich buffy coats from healthy blood donors, by a density gradient centrifugation over a Ficoll-Hypaque gradient. After washing, cells were resuspended in RPMI 1640 medium. For monocyte differentiation, peripheral blood mononuclear cells were allowed to adhere to plastic six-well plates for 2 h at 37 °C in a 5% CO_2 atmosphere in complete RPMI 1640 culture medium. After incubation, nonadherent cells were removed by washing twice with warm sterile phosphate-buffered saline (PBS, without Mg^{2+} or Ca^{2+}, pH 7.4). The adherent population was then scraped and viewed under light microscope. Viability was assessed as greater than 98% by trypan blue exclusion, and the cell population isolated was >87% CD14 positive as determined by flow cytometry. Cell viability was >90% even after 72 h of stimulation.

293/hTLR2–CD14 and 293/hTLR4A–MD2–CD14, HEK 293 cell lines stably transfected with TLR2–CD14 and TLR4–MD2–CD14 complex correspondingly, were obtained from Invivogen (San Diego, CA, USA). Adherent cells were pretreated with Trypsin–EDTA until the cell layer was detached and were resuspended in complete medium to a concentration of 1.8×10^6 cells/ml. Cell viability was examined by assessing the development of adhesion under an inverted microscope and by the trypan blue dye exclusion method and was determined to be >90%. Adherent cells, grown in 24-well tissue culture plates until they reached confluency, were stimulated with live bacteria or other stimulants.