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Evidence of *Brucella abortus* OPS dictating uptake and restricting NF-κB activation in murine macrophages

Jianwu Pei, **Joshua E. Turse**, and **Thomas A. Ficht***
Department of Veterinary Pathobiology, Texas A&M University and Texas Agricultural Experiment Station, College Station, TX 77843-4467

Abstract

Smooth *Brucella abortus* S2308 is virulent while rough derivatives are attenuated. Intracellular killing is often blamed for these differences. In the studies described, uptake kinetics and interaction of S2308 and S2308 *manBA*::Tn5 (CA180) rough mutants with macrophages were investigated. The results revealed that smooth *B. abortus* was rapidly internalized, achieving a maximum level in less than 5 minutes without additional uptake over the next 30 minutes. In contrast, continued uptake of the rough mutant was observed and only achieves a maximum level after 30 minutes. The results were confirmed by the differences in F-actin polymerization, lipid raft staining, early endosome colocalization and electron microscopic observations after smooth and rough *Brucella* infection. We also demonstrated for the first time that uptake of S2308, but not rough mutant CA180 was PI3-kinase and toll-like receptor 4 (TLR4) dependent. Differences in uptake were associated with differences in macrophage activation with regard to NF-κB translocation and cytokine production. These results provide evidence that the presence of *B. abortus* OPS dictates the interactions between *Brucella* and specific cell surface receptors minimizing macrophage activation and enhancing *Brucella* survival and/or persistence.

Keywords

Brucella; uptake; OPS; macrophage

1. Introduction

Brucella spp. are gram-negative, facultative intracellular bacteria that infect a variety of animals causing abortion and infertility. *B. melitensis, B. abortus* and *B. suis* also infect humans causing undulant fever. This worldwide zoonotic disease is endemic in many developing countries and is responsible for economic losses in livestock industries and public health concerns. The ability of this organism to cause disease in humans and animals following aerosol exposure has led to its inclusion on select agent and biodefense threat lists.

Macrophages and trophoblast cells are primary target cells in which the bacteria can multiply and cause persistent infection and abortion. The bacteria also infect a variety of non-

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*Corresponding author: Thomas A. Ficht, Department of Veterinary Pathobiology, Texas A&M University, College Station, TX77843-4467, Tel. (979)845-4118, Fax: (979)862-1088, tficht@cvm.tamu.edu

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professional phagocytes in culture, suggesting that *Brucella* have broad interactions with host cells [1]. *Brucella* invasion processes in epithelial cells and macrophages have been investigated. Early studies showed that M cells, macrophages and neutrophils ingest *Brucella* by zipper-like phagocytosis [2]. Rittig et al. (2001) recently confirmed that human monocytes and epithelial cells engulf *B. melitensis* and *B. suis* via conventional zipper-type mechanisms [3]. Invasion of HeLa cells by *B. abortus* requires small GTPase of the Rho subfamily [4] and requires energy input from the host cells [5]. It was also observed that *Brucella* invade macrophages through lipid raft microdomains [6]. In both epithelial cells and macrophages F-actin polymerization is involved in ingestion of *Brucella,* because inhibition of actin polymerization abolishes bacterial uptake [4,5,7].

Possible host cell surface receptors for *Brucella* have been recently identified [8-10], but the role of cellular prion protein in *Brucella* uptake remains questionable [11]. *Brucella* virulence factors involved in bacterial invasion have not been clearly defined. It has been reported that BvrS/BvrR two component regulatory system is important to *Brucella* penetration of professional and non-professional phagocytic cells [12]. The roles of type IV secretion system (T4SS) in *Brucella* invasion have been investigated and it is generally accepted that the T4SS is not involved [13]. More recent study showed that BMEI0216 gene of *B. melitensis* is required for internalization in HeLa cells [14]. Essential genes encoding invasins and adhesins have been identified by *Brucella* genome analysis, but those molecules have not been investigated on *Brucella* surface [15]. *Brucella* LPS has been recognized as an important virulence factor involved in bacterial adhesion and internalization [16]. Recent studies revealed that smooth *Brucella* invade phagocytes at lower number compared with rough organisms, and inhibit phagocyte apoptosis [17,18]. In contract, *Brucella* rough strains attach and invade macrophages at higher efficiency and activate macrophage [6,17,19,20]. However the mechanisms behind this have not been fully investigated.

In this study, the internalization processes and interaction of smooth and rough organisms with murine macrophages were monitored by a variety of methods. The results indicate that OPS dictates *Brucella* uptake by macrophages, and in doing so alters the outcome of infection by restricting macrophage activation.

2. Materials and Methods

2.1. Bacteria strains and growth conditions

Bacteria used in this experiment include *B. abortus* virulent S2308 strain and rough mutant S2308 *manBA*::Tn5 (CA180) [21]. The bacterial cultures were prepared as previously described [19].

2.2. Reagents

Mouse anti-*Brucella* and goat anti-*Brucella* sera were prepared in our laboratory. Donkey anti mouse IgG Alexa Fluor 594, donkey anti mouse IgG Alexa Fluor 488, donkey anti goat IgG Alexa Fluor 488, donkey anti goat IgG Alexa Fluor 594 and Alexa Fluor 488-phalloidin were purchased from Molecular Probes (Eugene, OR). Goat anti EEA1 and goat anti-NF-κB p65 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cholera toxin B subunit-FITC and wortmannin were obtained from Sigma (St. Louis, MO).

2.3. Cell culture and infection

Murine macrophage-like cell J774.A1 (ATCC, TIB-67) was grown in complete Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FBS and 0.1 mM non-essential amino acids (complete DMEM). The cells were passaged every 3 to 5 days and discarded after passage 15. Bone marrow derived macrophages (BMDM) were prepared from C3H/HeJ ($TLR4^{d/d}$) and

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C3H/HeOuJ ($TLR4^{+/+}$) mice as previously described [22]. Macrophages were seeded in 24-well or 6-well plates one day prior to infection with *Brucella* [19].

2.4. Immunofluorescent assay

J774.A1 cells cultured on glass coverslips with a thickness of 0.17 mm (Fisher Scientific, Pittsburgh, PA) were infected with S2308 or CA180 at an MOI of 10 as described previously [19]. The infected cells were fixed with 3% (w/v) paraformaldehyde immediately after a 5-minute centrifugation, and following 5, 10, 20, and 30 minutes incubations. To monitor bacterial infection within the first 5 minutes, J774.A1 cells cultured on coverslips were chilled on ice for 20 minutes. The cells were inoculated with S2308 and CA180 at 10 MOI, and centrifuged at 4°C for 5 minutes. Medium was replaced with pre-warmed complete DMEM (37°C) and the cells were fixed at 0.5, 1, 2, 3, 4, and 5 minutes.

For differential staining of intracellular and extracellular bacteria, fixed cells on coverslips were stained with a double antibody labeling procedure and observed as previously described [19]. At least 200 cells were detected to obtain each data.

To detect NF-κB translocation, J774.A1 macrophages cultured on coverslips were infected with S2308 or CA180 as described above. The cells treated with *E. coli* LPS (100 ng/ml) were used as positive control. The infected and control cells were fixed at 1 h p.i. and stained with goat anti p65 (1:500) and mouse anti *Brucella* (1:1000). NF-κB translocation and *Brucella* internalization was revealed by staining with donkey anti goat IgG Alexa Flour 594 and donkey anti mouse IgG Alexa Flour 488 (1:1000), respectively.

2.5. Drug treatment of macrophages before infection

To determine the roles of phosphoinositide 3-kinase (PI3-kinase) in the uptake of smooth and rough *B. abortus,* J774.A1 macrophages were treated with 100 nM of wortmannin for 30 minutes prior to infection. The cells were washed with complete DMEM before infection with S2308 and CA180 at an MOI of 100. Uptake of smooth and rough *Brucella* by the treated cells was determined using the gentamicin protection assay [19].

2.6. Cytokine ELISA

J774.A1 cells cultured in 24-well plates were infected with S2308 and CA180 at an MOI of 100 as described above. Cell culture supernatants were collected at various time points and cytokine levels in the supernatants were detected using sandwich ELISA kits (PeproTech Inc., Rocky Hill, NJ) following the manufacture's instruction.

2.7. Electron microscopy

J774.A1 cells cultured on glass coverslips or in 6-well plates were infected with S2308 and CA180 as described above. The infected cells were fixed at various time points as previously described [23] and processed at Image Analysis Laboratory in College of Veterinary Medicine and Biomedical Sciences, Texas A&M University. Bacterial invasion was observed with JSM 6400 scanning electron microscopy in Electron Microscopy Center, Texas A&M University or with Carl Zeiss High Resolution Electron Microscope (EM 10 CA) at the Image Analysis Laboratory in the College of Veterinary Medicine and Biomedical Sciences, Texas A&M University.

2.8. Statistical analysis

Statistical significance was determined using Student's *t*-test. A P value of < 0.05 was considered significant.

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3. Results

3.1. *B. abortus* OPS affects binding and internalization

It has previously been reported that the uptake of rough organisms greatly exceeds that observed for smooth strains [6,17,19], and current experiments were conducted to reveal the dynamics of internalization. A double antibody labeling procedure was employed to differentiate external bacteria (red) from internalized organisms (green) (data not shown). In agreement with previous reports [6,17,19], CA180 has greatly elevated binding capacity compared with S2308. The dynamics of internalization were determined according to the following criteria: i) fraction of cells containing internalized bacteria; ii) average bacterial burden per infected cell; and iii) fraction of cell-associated bacteria that was internalized (Fig. 1). Using this approach it was demonstrated that the uptake of smooth organisms by J774.A1 cells was optimal within 5 minutes of exposure, while rough organisms take up to 35 minutes to be optimally internalized (Fig. 1). The fraction of the cells infected with S2308 reached 5.25 ± 3.32% at 5 minutes p.i. and remained unchanged up to 35 minutes p.i. However, the fraction of cells infected with CA180 was 31.42 ± 1.53% by 5 minutes p.i., and increased to 94.95 ± 3.04% by 15 minutes p.i. (Fig. 1A). The average number for S2308 remained about one bacterium per infected cell between 5 and 35 minutes p.i., while the number for the CA180 steadily increased from 1.41 ± 0.23/cell at 5 minutes to 5.62 ± 0.12/cell at 35 minutes p.i. (Fig. 1B). Analysis of the infected cells revealed that 80.27 ± 13.12% of the cell-associated smooth *Brucella* were internalized within 10 minutes. In contrast, only 21.1 ± 9.05% of cell associated rough *Brucella* were internalized at 10 minutes and by 25 minutes the macrophages had internalized 59.15 ± 1.63% of cell-associated rough organisms (Fig. 1C). These results confirm that *Brucella* OPS suppressed bacterial binding and uptake by macrophages, and reveal for the first time that the internalization of smooth *Brucella* S2308 by macrophages occurs much faster reaching saturation within 5 minutes, while rough *Brucella* mutant CA180 need more than 35 minutes.

To further investigate the kinetics of internalization of smooth *Brucella*, J774.A1 macrophage cells were chilled on ice prior to infection and fixed within 5 minutes of infection with S2308 and CA180, and intracellular and extracellular bacteria were enumerated as described above. More than 80% of the cell associated S2308 invaded the macrophage in less than 5 minutes, while only 8.26% of the CA180 was internalized (Fig. 1D). This rapid invasion by smooth organisms made visualization of the invasion processes using scanning electron microscopy difficult (Fig. 1E), while the rough *Brucella* can be easily detected by 5 minutes p.i. (Fig. 1F) and still be detected at 20 minutes p.i. (Fig. 1G). Transmission electron microscopy observation confirmed that smooth *Brucella* were internalized by five minutes (Fig. 1H, 1I). In contrast, most of the rough organisms still attached to cell surface or invading the cells (Fig. 1J, 1K).

Actin polymerization has been identified as an essential part of the entry mechanism in phagocytosis of pathogens. Contact between *Brucella* and macrophages or HeLa cells induce transient local F-actin recruitment [4,7]. To further determine the role of *Brucella* OPS in induction of F-actin polymerization, J774.A1 macrophages infected with *B. abortus* S2308 and the rough mutant CA180 was monitored by phalloidin Alexa Fluor 488 staining (Fig. 2). Both smooth and rough *B. abortus* contact with the macrophages elicited F-actin polymerization (Fig. 2A). Staining of the infected cells fixed at different time points indicated that both smooth and rough *Brucella* colocalized with F-actin at very early stages of infection and quickly depolymerized. More than 90% of the smooth organisms colocalized with F-actin by 30 seconds p.i. which dropped to 20% by 5 minutes p.i. (Fig. 2B insert). In contrast, CA180 recruited F-actin slowly, with a maximum colocalization of 70% detected by 5 to 10 minutes p.i. (Fig. 2B), which decreased gradually to 25% by 35 minutes p.i. These results suggest a role for *Brucella* OPS in restricting overall binding and uptake of the organism, while enhancing the kinetics of internalization.

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Early endosomes are cellular compartments that receive and sort endocytosed material to late endosomes and lysosomes. To examine whether the early interactions of *Brucella* with the endocytic compartment was changed by OPS, J774.A1 macrophages were infected with S2308 and CA180. Staining of the infected macrophages with early endosome associated antigen (EEA1) revealed that both *Brucella* smooth and rough organisms invade macrophages through early endosomes (Fig. 2C). Examination of the infected cells fixed at various time points indicated that the interactions were transient. However, smooth *Brucella* S2308 entered early endosomes faster than the rough mutant CA180 (Fig. 2D), which is in agreement with the uptake kinetics described above.

3.2. Smooth *Brucella* uptake by macrophages is associated with lipid rafts

Recent studies have shown that lipid rafts or caveolae play an important role in bacterial entry. Lipid raft-associated molecules, such as cholesterol, ganglioside GM1, and glycosylphosphatidylinositol (GPI)-anchored protein, are selectively incorporated into macropinosomes containing *Brucella*. Among those molecules, membrane cholesterol and ganglioside GM1 have been shown to be important in establishing *Brucella* entry and infection [24]. Methyl-β-cyclodextrin or filipin treatments of J774.A1 cells inhibited uptake of smooth *Brucella* in a dose dependent manner, while the treatments had limited effect on the uptake of rough *Brucella* [6]. In the current report, colocolization of *Brucella* with lipid rafts was visualized using fluorescent microscopy. J774.A1 cells infected with S2308 and CA180 were stained with goat anti-*Brucella* antibody. Colocalization of the bacteria and GM1 was determined after staining with CTB-FITC conjugate and donkey anti goat IgG Alexa Fluor 594. In agreement with previous report [6], S2308 but not CA180 colocalized with GM1 (Fig. 3A). Maximum colocalization with S2308 is detected at 3 min p.i. and rapidly declines (Fig. 3B), which is consistent with rapid internalization and trafficking of this smooth organism .

3.3. Effect of PI3-kinase inhibitors on the invasion of smooth and rough *B. abortus*

It has been shown that PI3-kinase plays an important roles in phagocytosis and phagosome trafficking [25]. Reports of the effect of PI3-kinase on *Brucella* uptake have been controversial. Guzman-Verri et al. reported that PI3-kinase is involved in the uptake of *Brucella* S2308 by HeLa cells [4], while others showed that wortmannin (PI3-kinase inhibitor) treatment did not inhibit wild type *Brucella* uptake [26,27]. To determine the roles of PI3-kinase in the uptake of smooth and rough *B. abortus*, J774.A1 macrophages were treated with 100 nM of wortmannin before *Brucella* infection. Wortmannin treatment significantly inhibited the uptake of S2308 strain, but had limited effect on the uptake of the rough mutant (Fig. 4). These results demonstrated that PI3-kinase activity is important for the uptake of smooth *Brucella*, but appears to play a limited role (if any) in the uptake of rough mutants.

3.4. TLR4 is important for smooth *Brucella* uptake in murine BMDM

Signaling through TLRs has been shown to activate macrophages including enhanced bacterial phagocytosis and phagosome maturation, nucleus translocation of NF-κB and proinflammatory cytokine production [28]. To determine the role of TLR4 in *Brucella* invasion, uptake of smooth and rough *B. abortus* was evaluated in BMDMs from C3H/HeJ and C3H/HeOuJ mice. The results revealed that the defect in TLR4 resulted in almost 75% decrease in the internalization of smooth *B. abortus* S2308: 1×10^6 CFU/well in HeJ BMDM vs. 3.8×10^6 CFU/well in OuJ macrophages (Fig. 5A). In contrast, the defect in TLR4 had no apparent effect on internalization of rough mutant CA180: 1.37×10^7 CFU/well in HeJ BMDM vs. 1.38×10^7 CFU/well in OuJ BMDM (Fig. 5B). These results indicate that TLR4 is important for smooth *B. abortus* internalization in macrophages. The failure to significantly affect the internalization of rough mutant CA180 is consistent with the observations that smooth and rough *B. abortus* are internalized via different pathways.

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3.5. Macrophages are activated by rough, but not smooth *Brucella* infection

In order to elaborate on the mechanistic basis for enhanced uptake of rough organisms, the activation state of macrophages was evaluated. Translocation of NF-κB p65 was monitored 1 h p.i. using fluorescence microscopy. In these experiments, nucleus translocation of NF-κB was easily detected in J774.A1 macrophages exposed to rough organisms at an MOI of 100 (Fig. 6A) and an MOI of 10 (data not shown). In contrast, no translocation was evident in cells exposed to smooth organisms at an MOI of 100 (Fig. 6B). *E. coli* LPS (100 ng/ml) induced strong translocation of p65 (Fig. 6C), while no translocation was detected in untreated cells (Fig. 6D). In order to verify the overall activation state of these macrophages, cell culture supernatants collected at 8, 24, 48 and 72 h p.i. were examined for IL1-α and TNF-α production using sandwich ELISA. The results revealed that rough *Brucella* stimulated high levels of TNF-α (Fig. 6E) and IL1-α (Fig. 6F) production compared with S2308, indicating that macrophages were activated after rough *Brucella*, but not smooth *Brucella* infection.

4. Discussion

As an important innate immune component and primary target cell of *Brucella*, macrophage plays a central role in the pathogenesis of brucellosis. Interaction of the pathogen with macrophages determines the outcome of infection [29]. Unlike other pathogenic bacteria, *Brucella* sp. do not have classical virulence factors. Their virulence is determined by their abilities to invade, survive and reach the replicative niche in the host cells [16,30]. OPS is considered an important virulence factor in this organism [6,21,31,32]. The current study provides more evidence that *Brucella* OPS is essential for uptake by murine macrophages leading to a productive infection. With OPS, smooth *Brucella* S2308 invade macrophages via lipid rafts, which requires PI3-kinase and TLR4 signal and directs intracellular trafficking through a unique pathway to the replication niche [16]. Without OPS, rough *Brucella* mutant CA180 invade macrophages through a different pathway and are either destroyed as a result of macrophage activation or released prematurely as a result of cell death [17,19].

The internalization dynamics of S2308 and its rough derivative CA180 demonstrated that OPS limited overall binding and enhanced internalization dynamics. Uptake of smooth strains in macrophages reached saturation faster than the uptake of rough strains. The results were confirmed by F-actin polymerization, colocalization of the bacteria with EEA1 and electron microscopic observations. Saturated internalization was achieved within 3 minutes for S2308 and did not change for next 30 minutes, which has not been previously demonstrated [7]. In contrast to smooth bacteria, rough mutant CA180 invaded the cells for extended periods. The different internalization dynamics resulted in significant difference in percentages of infected cells by these organisms (Fig. 1A). One interpretation of these results may be that OPS blocks the interaction between *Brucella* and macrophage. Without OPS expression, bacterial membrane proteins are more exposed [33]. This hypothesis is supported by our recent observation that LPS isolated from *B. melitensis* 16M inhibits latex bead uptake in macrophage (J. Pei and T.A. Ficht, unpublished data). Another possibility is that loss of OPS expression may affect the conformation and function of various surface proteins [34]. In these cases, interactions between rough *Brucella* and macrophages could be enhanced. The third interpretation is that changes occur in cells following smooth *Brucella* infection or contact with the cells that restrict additional uptake. However, such changes (if they occur) do not prevent rough *Brucella* uptake in mixed infection with smooth and rough organisms, or in super-infection by rough *B. abortus* after smooth *Brucella* infection (data not shown). These results appear to support the hypothesis that *Brucella* smooth and rough strains invade macrophage via different pathways [6,35]. Furthermore, increased uptake is observed at elevated MOIs and prolonged infection time (data not shown). The fourth possibility is that *Brucella* infection, especially rough *Brucella* infection activates the macrophages and up-regulate receptor

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expression [36]. This assumption is supported by the recent report that the presence of smooth or rough *Brucella* LPS decreased smooth *Brucella* uptake, which suggested that, as a legand, LPS compete with *Brucella* cells for binding sites on macrophages [10,20]. The fact that smooth organisms exhibit increased uptake at either elevated MOIs or as a result of prolonged infection time is most likely an artifact resulting from the presence of spontaneous rough mutants in the culture or simply due to the overwhelming numbers of organisms interacting with the cell, and such experimental conditions should be avoided.

Although S2308 and rough mutant CA180 invade macrophages via different pathways, transient early endosomal association appeared to be similar, suggesting that the organisms start their intracellular trafficking by phagocytic pathway. The difference in smooth and rough *Brucella* association dynamics is due to the difference in uptake dynamics.

Phagocytes express a variety of cell surface receptors that participate in bacterial recognition and internalization. The cell receptors and molecular mechanisms involved in non-opsonized *Brucella* uptake have not been clearly identified, although sialic acid-containing molecules and class A scavenger receptor may serve as receptors as recently reported [8,10]. It has also been reported that opsonized *Brucella* are internalized via complement and Fc receptors in macrophages and monocytes [16]. However, the participation of such pathways in the establishment or persistence of infection remains to be elucidated. In the current report, *B. abortus* interaction with target cells was evaluated in the absence of opsonization.

Phagocytosis is extremely complex and many cell receptors, signaling molecules and pathways are involved. In terms of bacterial internalization, different microbes may recognize different receptors, activate different signaling pathways, and trigger diverse cellular processes such as cytoskeletal rearrangement, membrane ruffling, cytokine and chemokine production, and microbial killing mechanisms. Among these molecules, PI3-kinase is known to be involved in phagocytosis and phagosome maturation [25]. However, controversial results have been reported regarding the role of PI3-kinase in *Brucella* phagocytosis [4,26,27]. Our results revealed that PI3-kinase is essential for smooth, but not rough *B. abortus* uptake by murine macrophage J774.A1, suggesting that uptake mechanisms of smooth and rough *Brucella* are different in these cells.

Recent studies showed controversial results regarding the role of TLR signals in bacteria uptake and phagosome maturation [28,37]. Blander et al. reported that TLR signals are essential for bacteria uptake and bacterial phagosome maturation in macrophages [28]. However, Yates and Russell demonstrated that TLR signals are not required for phagosome maturation [37]. Our current study revealed that the uptake of smooth but not rough organisms is TLR4-dependent. The link between TLR4 and lipid raft formation has been documented [38] and the impact of the loss of TLR4 signal is suspected to be a direct result of the failure to form lipid rafts necessary for the uptake of smooth *Brucella*. Another finding in this study is that smooth *B. abortus* failed to activate NF-κB translocation to the macrophage nucleus and is undoubtedly responsible for the observed difference in production of cytokines and nitric oxide after smooth and rough *Brucella* infection [20,35]. This is consistent with the stealth nature of smooth *Brucella* [22].

In conclusion, we have provided evidence that *Brucella* OPS controls the organism uptake process and restricts macrophage activation to establish a replicating niche. In contrast, rough *Brucella* mutants invade macrophage via different pathways activating macrophage and leading to bacterial destruction [19,23].

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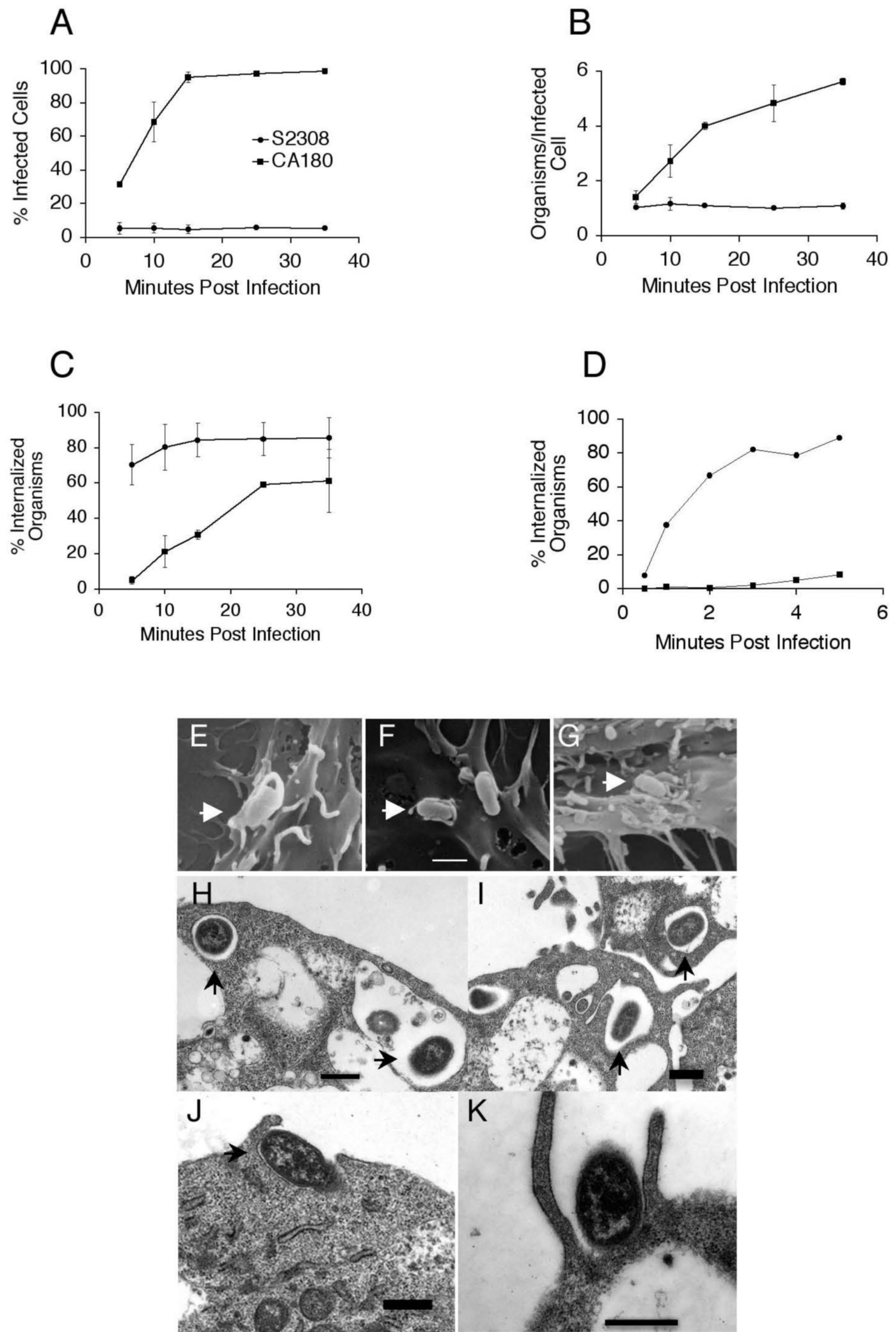

Fig. 1.

B. abortus invasion dynamics and electron micrographs in murine macrophages. The invasion of *B. abortus* smooth strain S2308 and the rough derivative CA180 was monitored over time using double antibody staining and electron microscopy. Percentage of the cells infected at indicated time points (A). Average internalized bacteria per infected cell (B). A ratio (percent) of internalized bacteria to cell-associated bacteria (C). The results are means ± SD of three independent experiments. A ratio (percent) of internalized bacteria to cell-associated bacteria at early stages (less than 5 minutes) of infection (D). The data is a representative of two experiments with similar results. Scanning electron micrograph of macrophages infected with S2308 and fixed at 5 minutes p.i. (E), or infected with CA180 and fixed at 5 minutes (F) or 20

minutes p.i. (G). Transmission electron micrograph of macrophages infected with S2308 (H, I) and CA180 (J, K) and fixed at 5 min p.i. Bar = 1 μM.

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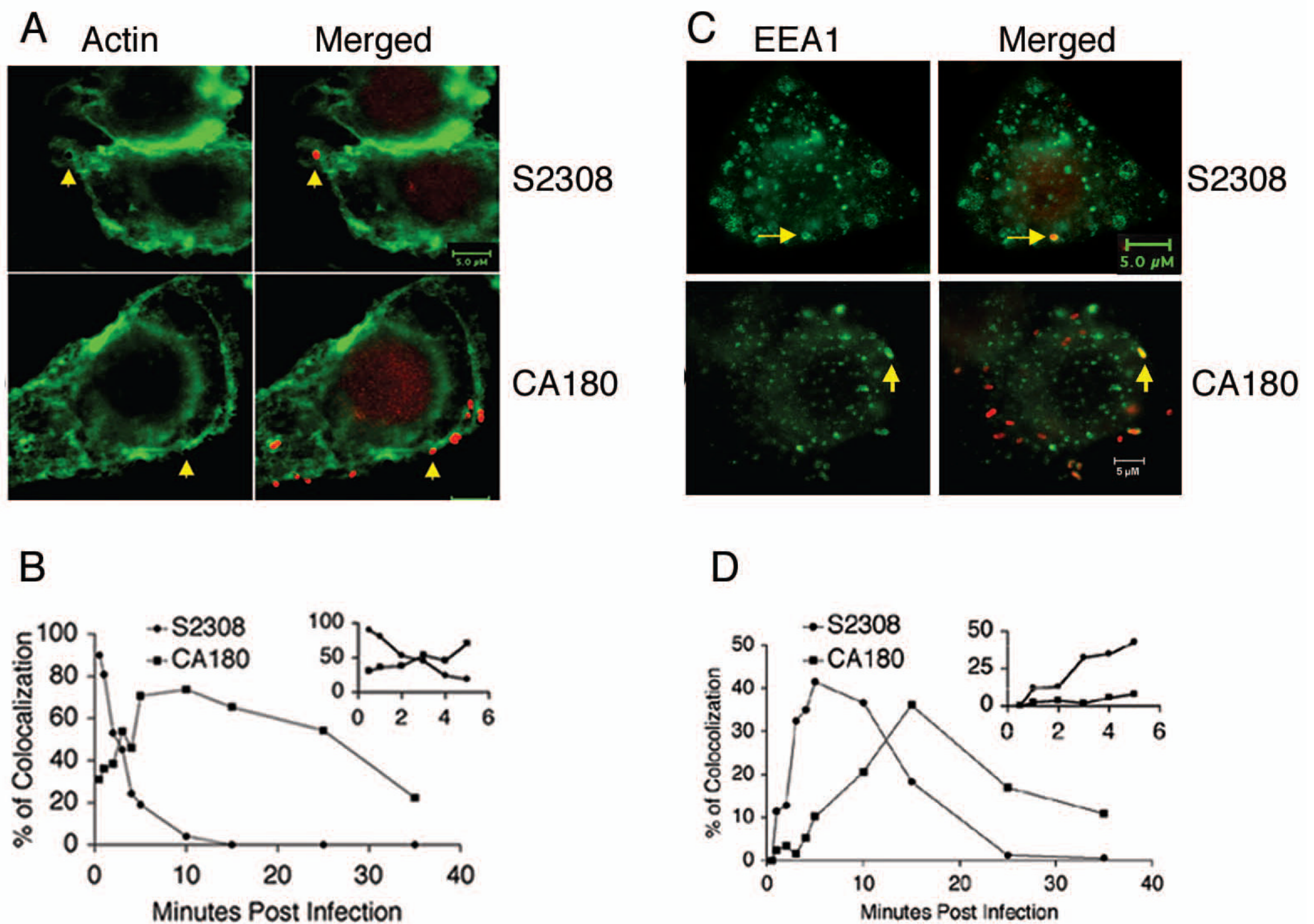

Fig. 2.
F-actin polymerization and early endosome marker colocolization during *B. abortus* invasion. J774.A1 macrophages cultured on coverslips were infected with S2308 and CA180 at an MOI of 10 and fixed with 3% (w/v) paraformaldehyde at selected time points. The bacteria (red) were stained with mouse anti *Brucella* serum and detected with donkey anti mouse IgG Alexa Fluor 594. F-actin (A) or EEA1 (C) was detected as described in the Materials and Methods. The dynamics of *Brucella* colocalization with F-actin (B) or EEA1 (D) were revealed in time course. The insert shows the dynamics in early stage of the infection (5 minutes or less). The data shown is a representative of two experiments with similar results.

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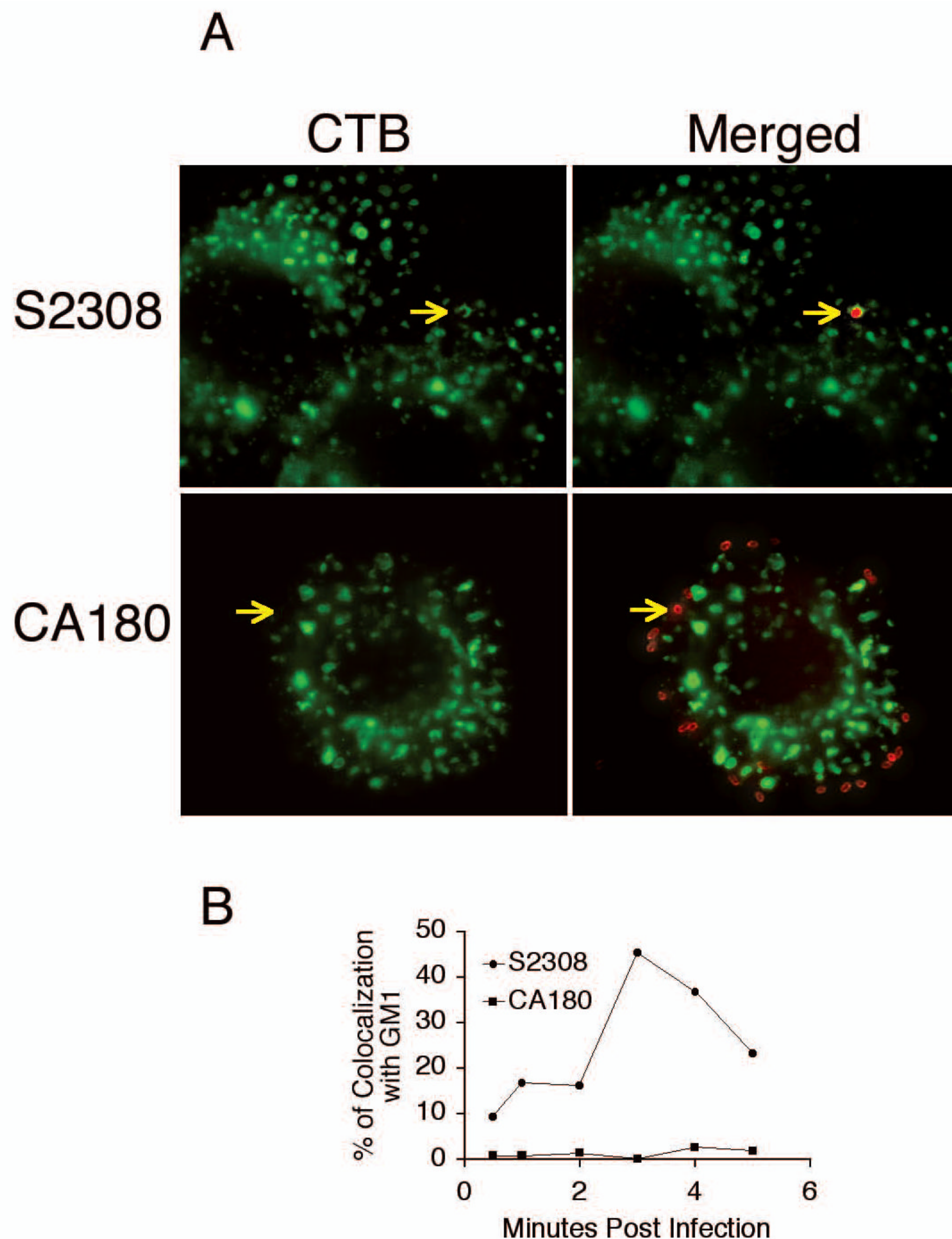

Fig. 3.

Brucella smooth strain invades macrophages using lipid rafts as revealed by GM1 colocalization. J774.A1 macrophages cultured on coverslips were infected with S2308 and CA180 at an MOI of 10 and fixed with 3% (w/v) paraformaldehyde at various time points. (A) The bacteria (red) were stained with mouse anti *Brucella* serum and detected with donkey anti mouse IgG-Alexa Fluor 594. Ganglioside GM1 (green) was detected by cholera toxin B subunit-FITC staining. (B) Dynamics of *Brucella* colocalization with GM1. The result is a representative of two experiments with similar results.

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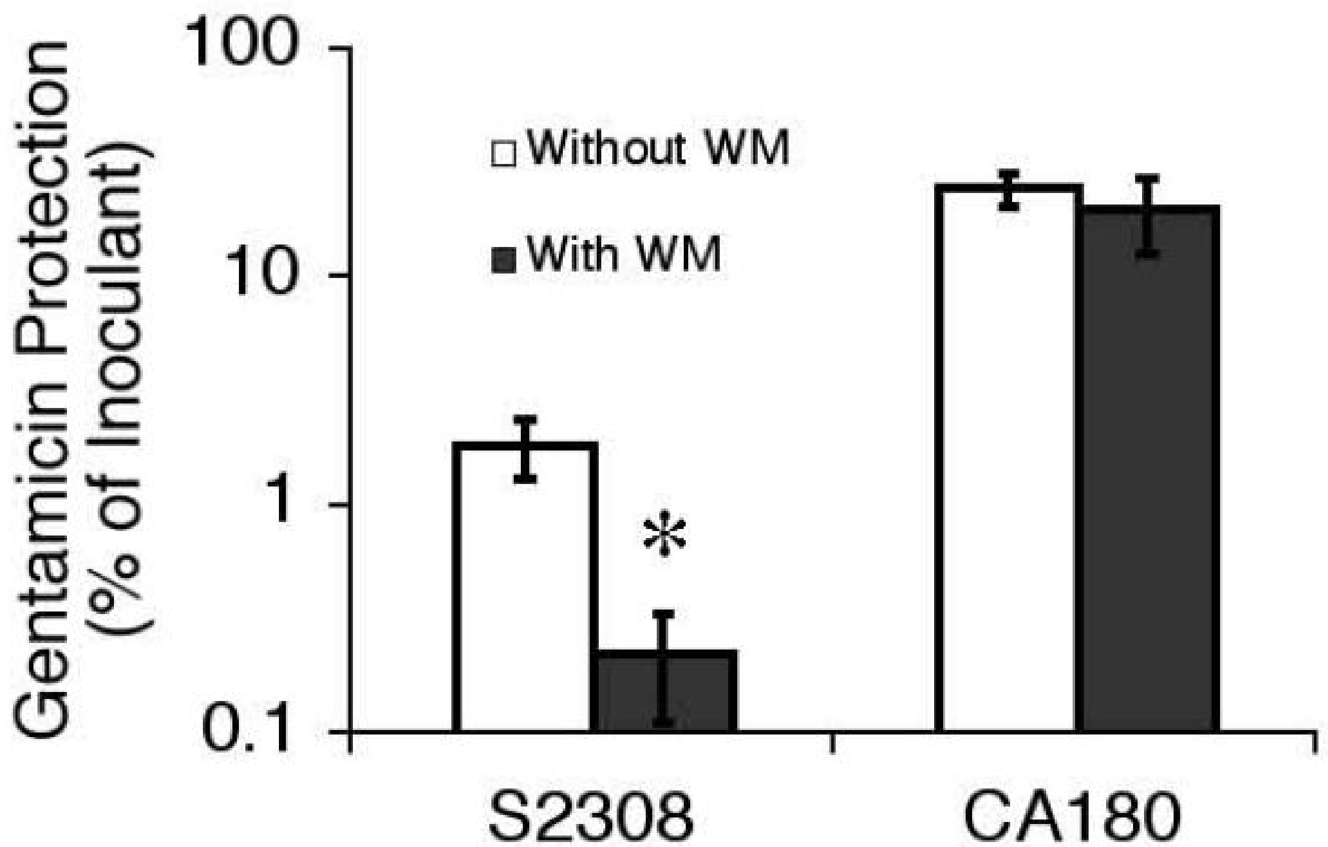

Fig. 4.

PI3-kinase in the uptake of *Brucella* in macrophages. J774.A1 was treated with 100 nM wortmannin for 30 minutes before infection and continuously thereafter. Gentamicin protection assay was used to monitor the uptake of S2308 and CA180 in the presence and absence of wortmannin. The data shown represent the means ± SD of three independent experiments. □$p<0.05$ when compared with untreated control.

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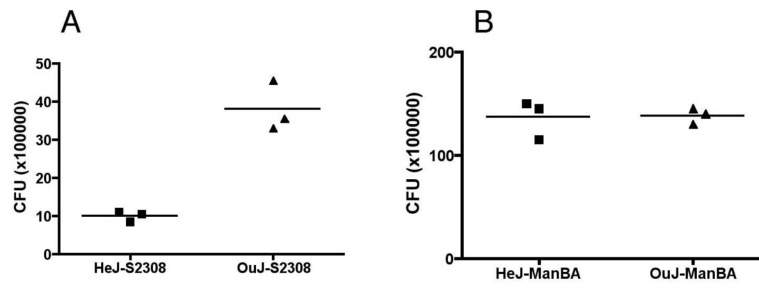

Fig. 5.

TLR4 is important for smooth *Brucella* uptake in murine macrophage. BMDMs cultured from (A) TLR4 deficient (HeJ) or (B) TLR4 sufficient control (OuJ) mice were infected with smooth and rough *Brucella* at an MOI of 100. Uptake of the bacteria was determined using gentamicin protection assay. The results are representatives of four experiments

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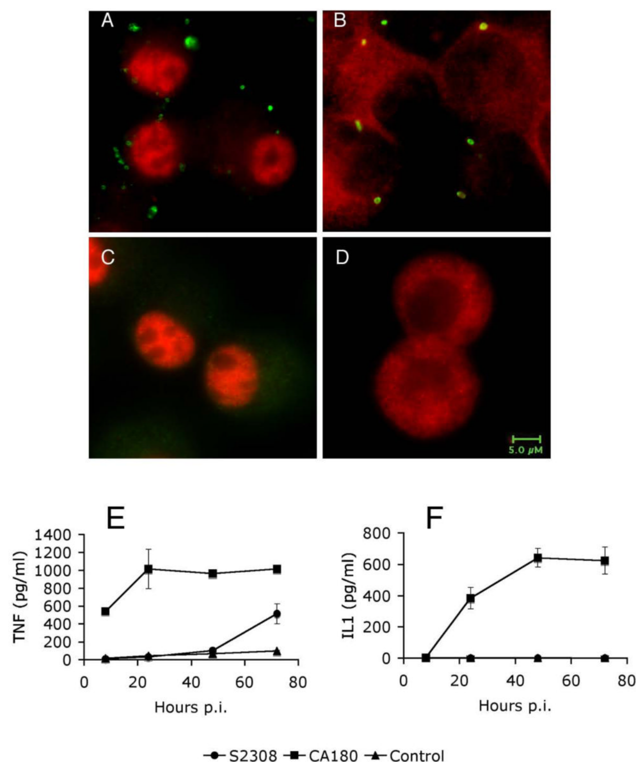

Fig. 6.

Rough *Brucella* infection induces macrophage activation. J774.A1 macrophages cultured on coverslips were infected with CA180 (A) or S2308 (B) at an MOI of 100. Control cells were treated with 100 ng/ml *E. coli* LPS (C) or left untreated (D). The cells were fixed with 3% (w/v) paraformaldehyde 1 h p.i. and detected for p65 (red staining) translocation and *Brucella* (green) using IX70 fluorescence microscopy (Olympus) after immunofluorescence staining. TNF-α (E) and IL1-α (F) were detected using sandwich ELISA after macrophages were infected with smooth and rough *B. abortus* at an MOI of 100. The supernatants were collected at 8, 24, 48 and 72 h p.i. The results (mean ± SD) are representatives of three experiments with similar results.