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Inflammatory response of TLR4 deficient spleen macrophages (CRL 2471) to *Brucella abortus S19* and an isogenic *ΔmglA* deletion mutant

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ABSTRACT

Brucellosis is a worldwide distributed zoonosis caused by members of the genus *Brucella*. One of them, *Brucella abortus*, is the etiological agent of bovine brucellosis. With the attenuated strain *B. abortus S19* a vaccine is available. However, both, virulence (safety) and the ability to induce a protective B and T cell response (efficacy) have to be tested in suitable assays before successful use in the field. For this purpose, several macrophage cell lines of various origins have been used while splenic macrophages are the preferred host cells *in vivo*.

We here characterized the *in vitro* response of the murine splenic macrophage cell line CRL 2471(I-13.35) to *B. abortus*. This cell line still depends on the presence of colony-stimulating factor 1 (CSF1) and is derived from LPS resistant (TLR4 deficient) C3H/HeJ mice. For infection the vaccine strain *B. abortus S19A* as well as the formerly described isogenic deletion mutant *B. abortus S19A ΔmglA 3.14* were used.

While numbers of viable bacteria did not differ significantly between the vaccine strain and the deletion mutant at 6 h post infection, a higher bacterial load was measured in case of the mutant at 24 h and 48 h after infection. This was also true, when IFNγ was used for macrophage activation.

A comprehensive gene expression profile of macrophages was analysed 6 and 24 h after infection by means of an RT-PCR based gene expression array. The mutant strain *B. abortus S19A ΔmglA 3.14* elicited a stronger cellular response of the splenic macrophages as compared to the parental vaccine strain. This was most prominent for the pro-inflammatory cytokines IL-1α, IL-1β, TNF-α and IL6 as well as for the chemokine ligands CXCL1, CXCL2, CXCL10, CCL2, CCL5, CCL7, CCL17 and the co-stimulatory molecules CD40 and ICAM1. While these differences were also present in IFNγ-stimulated macrophages, an addition of IFNγ after infection not only resulted in a dramatic increase of the translation of the afore mentioned genes but also resulted in the translation of IFNß1, IL12ß, MIP1α and β (CCL3, CCL4), NOS2 (and SOD2) and FAS.

Conclusion: The TLR4 deficient murine splenic macrophage cell line CRL 2471 was used for the first time for the characterization of macrophage-*Brucella* interaction to investigate the pre-immune phase of brucellosis *in vitro*. Typical pro-inflammatory cytokines and certain surface receptors were differentially induced by *B. abortus S19 A* and an isogenic *ΔmglA* deletion mutant *in vitro*. This model may be useful for further studies to characterize the inflammatory response of splenic macrophages to intracellular gram-negative bacteria avoiding cell responses to soluble LPS.

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1. Introduction

Bacteria of the genus *Brucella* cause chronic infections in both humans and a variety of animal species. The bacteria belong to the class of α-Proteobacteria, phylum Proteobacteria, family Brucellaceae in the order Rhizobiales. Their closest relatives are bacteria of the genera *Agrobacterium, Bartonella, Ochrobactrum*, and *Rhizobium* (Moreno et al., 1990).

Brucella does not bind complement nor produce any exotoxins and possess a special but less toxic LPS. With the exception of a Type IV secretion system only few other virulence factors are produced (Goldstein et al., 1992; Martirosyan et al., 2011). The major pathogenetic principal is their ability to adapt and survive inside of even activated macrophages and granulomatous inflammation is the pathological hallmark (Martirosyan et al., 2011). Since the classical work of Holland and Pickett, and subsequently Mackaness it is known that the induction of a potent T cell mediated immunity

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in vivo depends on viable bacteria (Holland and Pickett, 1958; Mackaness, 1964, 1967) and thymic (T-cell) deficient nude mice suffer from chronic infection. However, the bacterial and host factors determining immunogenicity are not well described.

In the context of our work to characterize *Brucella*-host-interactions (Hort et al., 2003), we previously characterized a spontaneous smooth small colony variant of *Brucella abortus S19* which demonstrates not only a reduced growth rate *in vitro* but also a less effective clearance from both spleens and livers of experimentally infected mice (Jacob et al., 2006). Using a differential approach to analyse mRNA-derived cDNA, we identified a molecular difference in the transcription of a gene predicted to encode for a formerly described galactoside transport ATP binding protein, *mglA*, in the small colony variant and generated an isogenic *mglA* deletion mutant (Jacob et al., 2006; Genbank acc. numbers DQ839241, EU402949 and EU41049; Jacob et al., 2012). According to Jiang et al. (2013), such ABC-Transporters may play an important role in the virulence of *Brucella* strains and can influence trafficking of *Brucella* to compartments associated with endoplasmic reticulum.

Macrophages are important host cells of virulent *Brucella* and host defence is initiated by the recognition of pathogen-associated molecular patterns (PAMP's). This is followed by the (weak) activation of intracellular signalling pathways and uptake of the bacteria. Toll-like receptors are known to be involved in this process (Campos et al., 2004; Oliveira et al., 2008; Oliveira et al., 2012; Gomes et al., 2012; Pei et al., 2012).

Macrophages used so far for *in vitro* investigation include primary macrophages (a) from the peritoneum of BALB/CByJ mice (Jones and Winter, 1992), (b) from the alveolar tract of C57BL/6J, B6J.CD45.1 and B6.hCD2-DsRed mice (Archambaud et al., 2010), (c) from the spleen of CBA/H mice (Riglar and Cheers, 1980), (d) from liver of C57BI/6 × DBA/2)F1 mice (Buiting et al., 1995), (e) brain (astrocytes) from BALB/C and C57BL/6 mice (Samartino et al., 2010), and (f) from bovine mammary glands (Harmon et al., 1988).

Commonly used permanent cell lines are J774A.1 (ATCC® TIB-67); RAW 264.7 (ATCC® TIB-71) (Eskra et al., 2003) and THP-1 (ATCC® TIB-202™) (Köhler et al., 2002). Since the spleen is the most heavily afflicted organ in *Brucella* infected mice, splenic macrophages were selected in this paper to further characterize the *Brucella*-macrophage interaction *in vitro*.

In order to identify cell responses to intracellular gram-negative bacteria, that are less dominated by an LPS/TLR4 interaction, we investigated the splenic macrophage cell line CRL 2471 (I-13.35), which has several most interesting characteristics including the lack of TLR4 but is not yet well characterized (Wilson et al., 1991; McCormack et al., 1992).

We therefore performed a comprehensive analysis of the *Brucella*-macrophage-interaction using RT-PCR array technology and cytological methods as well as two strains of *B. abortus S19* including the formerly described isogenic *ΔmglA 3.14* deletion mutant.

2. Material and methods

2.1. Bacterial strains and media

B. abortus S19A and *B. abortus S19 A ΔmglA 3.14* were obtained and grown in Trypticase Soy Broth (TSB) or on Trypticase Soy Agar (TSA) and subsequently stored as previously described (Hort et al., 2003).

2.2. Macrophage cell line

The cell line CRL 2471(I-13.35) was obtained from LGC Standards (Wesel, Germany) and is characterized by a high degree of similarities to the cell line RAW 264.7 (Wilson et al., 1991; McCormack et al., 1992; LeCureux et al., 2011). CRL 2471(I-13.35) cells are non-tumour forming adherent spleen macrophages. They were previously isolated from the spleen of an adult female mouse of the LPS low responder strain C3H/HeJ by Jackson Laboratories. Their growth in culture depends on colony stimulating factor 1 (CSF1). Expression of CD11b/CD18 (Mac1), MHC Class I, MHC Class II und CD115 (colony-stimulating factor 1 receptor (CSF1R) has been shown (Wilson et al., 1991). CRL 2471(I-13.35) cells were grown in tissue culture flasks or 24 well plates respectively in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) medium (Invitrogen, cat. number 41966) supplemented with 10% fetal calf serum and 2 mM/l L-glutamine at 37 °C in humidified air containing 5% carbon dioxide. Colony stimulating factor 1 (CSF1) was provided by 20% supernatant of cultivated Ladmac® cells ATCC® CRL 2420 (Sklar, 1985) and was present during the whole period of macrophage culture until the end of the experiment (*i.e.* also in the culture medium that was replenished).

2.3. Macrophage infection assay

Cells were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) in a large covered T175 cell culture flask and adherent cells were scraped using a cell scraper M (TPP, Switzerland). The cells were washed 2× with supplemented (10% fetal calf serum, 2 mM/l L-glutamine and 20% Ladmac® cell supernatant) DMEM medium without Penicillin/Streptomycin and the cell number was determined to be adjusted to 5×10^5/ml. The individual wells of a 24 well plate were filled with 1 ml of the cell suspension with and without 500 μm Fe^{2+}-sulphate (Sigma, Munich, Germany), respectively and incubated overnight at 37 °C and 5% CO_2.

The next day, the monolayer was examined for confluent growth. Subsequently, cells were infected with *B. abortus S19A* or *B. abortus S19A Δ mglA 3.14*. The infectious dose was 1×10^7 cfu/ml/well. The infection was stopped by washing the wells three times with 1.5 ml medium containing 10 μg/ml streptomycin. The subsequent culture was performed in the presence of medium containing 10% fetal calf serum, 2 mM/l L-glutamine and streptomycin which was changed every day. The cells were analyzed 6, 24 and 48 h after infection. At these time points, cells were washed 3 times with PBS and lysed in 0.5% deoxycholic acid. Finally they were plated in ten-fold dilutions on TSA agar to determine the number of viable intracellular bacteria.

In parallel supernatants were plated on TSA and cultivated for up to 4 days to check for extracellular growth of *Brucella*. Only experiments with *Brucella*-free supernatants (no growth on TSA after 4 days of culture) were used for analysis. Statistical analysis was done by means of Graph Pad Prism 5.04 software (Man–Whitney-*U* test).

In experiments using IFNγ, 100 U/ml IFNγ were added 2 h post infection. The medium which was added in the manner described above was replaced each 24 h post-infection still containing IFNγ in the IFNγ related experiments.

The cells were analyzed for 6, 24 h and 48 h after infection. The CRL 2471 cells with IFNγ treatment were handled in the same way as those without IFNγ treatment to determine the number of viable intracellular bacteria.

2.4. Infection of mice

For histological investigation, spleens from mouse experiments published before were used (Jacob et al., 2012). In short: Female 10–12 week-old BALBc mice were used. Mice were raised in the breeding facilities of Charles River WIGA (Sulzfeld, Germany) under specified pathogen-free conditions. The experiments were performed in the BSL3 facilities of the Robert Koch-Institute using an

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