Coxiella burnetii Infects Primary Bovine Macrophages and Limits Their Host Cell Response

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Although domestic ruminants have long been recognized as the main source of human Q fever, little is known about the lifestyle that the obligate intracellular Gram-negative bacterium *Coxiella burnetii* adopts in its animal host. Because macrophages are considered natural target cells of the pathogen, we established primary bovine monocyte-derived macrophages (MDM) as an *in vitro* infection model to study reservoir host-pathogen interactions at the cellular level. In addition, bovine alveolar macrophages were included to take cell type peculiarities at a host entry site into account. Cell cultures were inoculated with the virulent strain Nine Mile I (NMI; phase I) or the avirulent strain Nine Mile II (NMII; phase II). Macrophages from both sources internalized NMI and NMII. MDM were particularly permissive for NMI internalization, but NMI and NMII replicated with similar kinetics in these cells. MDM responded to inoculation with a general upregulation of Th1-related cytokines such as interleukin-1β (IL-1β), IL-12, and tumor necrosis factor alpha (TNF-α) early on (3 h postinfection). However, inflammatory responses rapidly declined when *C. burnetii* replication started. *C. burnetii* infection inhibited translation and release of IL-1β and vastly failed to stimulate increased expression of activation markers, such as CD40, CD80, CD86, and major histocompatibility complex (MHC) molecules. Such capability of limiting proinflammatory responses may help *Coxiella* to protect itself from clearance by the host immune system. The findings provide the first detailed insight into *C. burnetii*-macrophage interactions in ruminants and may serve as a basis for assessing the virulence and the host adaptation of *C. burnetii* strains.

*Coxiella burnetii*, a Gram-negative obligate intracellular bacterium, is the causative agent of Q fever, a widely distributed zoonoanthroponosis. After an incubation time of 2 weeks, Q fever can manifest as an acute, self-limiting flu-like illness with complications such as pneumonia and hepatitis. Chronic disease occurs more rarely and presents as, e.g., endocarditis or fatigue syndrome (1). Pregnant women are at high risk for developing chronic Q fever, which can result in an adverse pregnancy outcome with, e.g., spontaneous abortion or fetal death (2).

The common sources for transmission of *C. burnetii* to humans are domestic ruminants. Infection in animals, called cxiellosis, is mostly apparent, but chronic infection may lead to abortions with rates ranging from 5 to 91% in infected small-ruminant flocks (1). Placental membranes of infected animals contain up to 10⁷ organisms/g of tissue (3). Humans become infected by aerosols derived from contaminated abortion material, birth products, urine, or feces. Many Q fever outbreaks can be traced back to *C. burnetii*-infected small ruminants (4). The potential risk arising from cattle has been discussed repeatedly because these animals excrete, more than do small ruminants, huge amounts of *C. burnetii* through milk over a long time (5). Oral transmission by ingestion of contaminated raw milk or dairy products could lead to seroconversion but reportedly has resulted so far only in a few cases of Q fever (5–7).

The threat to humans is primarily determined by the number of *C. burnetii* particles shed from infected ruminants. The magnitude of shedding eventually relies on the interaction with placental, intestinal, and mammary epithelia (8). Knowledge about tissue dissemination of *C. burnetii* in ruminants is sparse, but infection studies in pregnant goats revealed that *C. burnetii* has a strong tropism for placental trophoblasts (9). However, the initial contact of *C. burnetii* with the animal host at the entry site, e.g., the respiratory mucosa, and the characteristics of the subsequent host response are decisive for controlling pathogen replication. A *C. burnetii* infection initiates humoral and cellular immune responses (10). Peripheral blood mononuclear cells (PBMC) from infected goats respond to *C. burnetii* restimulation with proinflammatory signals, e.g., upregulation of tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ). Despite activation of innate immune effectors, the development of adaptive immune responses upon natural infection appears to be insufficient, as goats are not capable of clearing a *C. burnetii* infection (11).

In the lung and placenta of infected animals, *C. burnetii* antigens are often found in macrophages (12–14). Members of the mononuclear phagocyte system are generally regarded as the main target cells for *C. burnetii*. The ability of such cells to rapidly respond to internalization of pathogens is central for their antibacterial responses (15). In murine and human macrophages, *C. burnetii* replicates in parasitophorous vacuoles (PVs) with lyosomal acidic characteristics (16). Infection in these cells induces an early proinflammatory response characterized by increased expression of cytokines such as interleukin-12 (IL-12), TNF-α, and IL-1β (17, 18). These mediators are important for recruiting other immune cells to the infection site, promoting pathogen clearance,
and development of adaptive immunity (15), essential for controlling *C. burnetii* infection in mice (19).

To develop novel measures for restricting the spread of *C. burnetii* in herds or their transmission to humans, alleged differences in the virulence of different *C. burnetii* strains must be considered. Different courses of natural and experimental infections in human patients and mice, respectively, imply that *C. burnetii* strains vary in their virulence properties, but an association with certain genotypes is weak at best. Gene mapping (20) and sequencing of the *C. burnetii* genome unveiled potential virulence genes based on sequence homologies. For example, the acute-disease antigen A (adaA) was described to be primarily present in strains isolated from acutely diseased patients (21). Despite an increasing number of effector proteins identified, their role as virulence factors during *C. burnetii* infection remains to be proven (22–25). Virulence of *C. burnetii* is strongly determined by phase variations similar to smooth-rough lipopolysaccharide (LPS) of enterobacteria (26).

Phase I strains possess a full-length LPS and are regarded as the naturally occurring, highly virulent form of *C. burnetii*. *Serial in vitro* passage of phase I *C. burnetii* leads to a conversion into avirulent phase II variants with truncated LPS. Compared to phase I, passage of phase I *C. burnetii* lacking adaA is described to be primarily present in strains isolated from acutely diseased patients (21). Despite an increasing number of effector proteins identified, their role as virulence factors during *C. burnetii* infection remains to be proven (22–25). Virulence of *C. burnetii* is strongly determined by phase variations similar to smooth-rough lipopolysaccharide (LPS) of enterobacteria (26).

Phase I strains possess a full-length LPS and are regarded as the naturally occurring, highly virulent form of *C. burnetii*. *Serial in vitro* passage of phase I *C. burnetii* leads to a conversion into avirulent phase II variants with truncated LPS. Compared to phase I, phase II variants lack certain sugars as part of the LPS O chain. Avirulent phase II strains are more efficiently internalized by host cells and cleared faster by the immune system than virulent phase I strains (27).

Lacking appropriate test systems, it is difficult to determine to what extent virulence properties beyond LPS affect the capability of *C. burnetii* to multiply and become shed by the reservoir host. Here we established an *in vitro* infection model with bovine monocyte-derived macrophages (MDM) and investigated bacterial replication and host cell cytokine responses after infection with virulent *C. burnetii* strain Nine Mile I (NMI) and avirulent strain NMII. For a better interpretation of key results, bovine alveolar macrophages were also infected. This is the first study to show that bovine MDM are a suitable model to explore the intracellular lifestyle of *Coxiella* in the cells of the natural host and to provide insights into the immune response evoked by *C. burnetii* infections upon first host contact.

**MATERIALS AND METHODS**

**Propagation, enumeration, and characterization of bacterial strains.** Chemicals were purchased from Sigma-Aldrich, Hamburg, Germany, unless otherwise stated. *C. burnetii* NMI (NMI strain RSA493) from the strain collection of the Institute for Hygiene and Infectious Diseases of Animals (IU [28]; NMI was propagated in mice, resolated, and subsequently passed 9 times in cell culture before being used in this study), and NMII (strain 439, clone 4; generously supplied by Anja Lührmann, Institute for Clinical Microbiology, Immunology and Hygiene, University Hospital Erlangen, Erlangen, Germany) were propagated in African green monkey kidney (BGM) cells and purified as described previously (29). Bacterial concentrations were determined by counting *C. burnetii*-like particles in Gimenez-stained smears using an Ortholux II microscope with a counting tube (Leitz-Leica, Mannheim, Germany). Briefly, suspensions were diluted 1:100 with sterile saline, and 10−μl aliquots were air dried on a 1-cm2 section of a slide and fixed with 100% methanol for 1 h. Slides were incubated for 6 min in 0.5 ml carboxyfluorescein working solution (1.5 mg/ml Neofuchsin [Merck, Hellepur, Denmark], 3 mg/ml phenol [Merck] in phosphate buffer). Slides were rinsed twice with water and counterstained with 0.5 ml mala- chite green working solution (8 g mala- chite green [Merck] in 1,000 ml distilled water) for 1 min. Bacterial concentration was calculated on baseline of *C. burnetii* particles counted in 100 small ocular squares at 787.5-fold magnification (30). The bacterial concentration was used for multiplicity of infection (MOI) determination. *C. burnetii* suspensions in NaCl solution were aliquoted (1 x 106 bacterial cells per ml) and stored at −80°C until further use. Immediately prior to inoculation, aliquots were thawed at ambient temperature and resuspended thoroughly for at least 1 min to separate bacterial agglutination. Relative abundances of full-length and truncated LPS molecules forming the cell envelope of the *C. burnetii* strains were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 to 20% Tris-glycine gel) and subsequent silver staining of the gels (31).

*Chlamydia psittaci* strain DC15, propagated and purified in BGM cells as described before (32), was used for control experiments.

**Preparation of bovine MDM and alveolar macrophages.** Bovine MDM were isolated following an established protocol (33–35). Briefly, a cirtated whole-blood sample (5:1 diluted with 3.8% sodium citrate solution) was centrifuged (2,380 x g, 20 min), and the buffy coat was collected. After several washing steps with phosphate-buffered saline (PBS)-EDTA buffer (PBS supplemented with 5.4 mM EDTA; 800 x g, 10 min), the remaining erythrocytes were lysed by incubation of the resuspended pellet in lysis buffer (8.26 g NaCl, 0.90 g NaHCO3, 0.037 g Na2EDTA, and 1,000 ml distilled water) for 10 min. Remaining cells were washed three more times (PBS-EDTA buffer; 300 x g, 4°C, 10 min) and layered onto Pancoll (PAN-Biotech, Aidenbach, Germany) for density centrifugation. Mononuclear cells were collected from the interphase, centrifuged at 800 x g for 10 min, and washed twice with 0.89% NaCl solution (300 x g, 10 min). Cells were adjusted to 4 x 105/ml in Iscove’s modified Dulbecco’s medium (IMDM) culture medium 1 (IMDM without phenol red, supplemented with 20% heat-inactivated fetal calf serum [FCS], 1% penicillin-streptomycin, 1% amphotericin B, 0.05% 100 mM β-mercaptoethanol). Twenty-five milliliters of this cell suspension was transferred to Teflon bags (VueLife Bags, American Fluoroseal Corp., Gaithersburg, MD, USA) and incubated for 7 days (37°C, 5% CO2). At the end of the incubation period, the cells were harvested and cultured in IMDM culture medium 2 (IMDM without phenol red, 2% FCS, 1% penicillin-streptomycin, 1% amphotericin B, 0.05% 100 mM β-mercaptoethanol). Cells were seeded into uncoated cell culture plates (Cellstar for suspension cultures; Greiner, Frickenhausen, Germany), unless otherwise stated. After 18 h of incubation, nonadherent cells, i.e., mostly lymphocytes, were carefully washed away with 0.89% NaCl solution, and adherent macrophages were left. Cell culture medium 3 (IMDM culture medium 2 devoid of antibiotics) was added to 1 to 2 further days before inoculation with *C. burnetii*. For each preparation, the cell composition of the culture was determined by fluorescence-activated cell sorter (FACS) analysis (see below). Cultures consisted of 81% ± 2.8% (mean ± standard deviation [SD] for 8 cultures) CD14+ cells with light scatter characteristics (size and granularity) of macrophages.

For the preparation of alveolar macrophages, bronchoalveolar lavage (BAL) fluid was extracted from the lungs of clinically healthy calves with a protocol described before (36). After a washing step, cells were harvested and cultured in IMDM culture medium 2. Cells were seeded into cell culture plates and incubated overnight for adherence. Cells were washed again, and the medium was changed to IMDM culture medium 3 1 to 2 days before inoculation with *C. burnetii*. Cultures consisted of 98% cells with morphological characteristics (size and granularity) of macrophages. All animal experiments were conducted according to the rules laid down in the German Animal Protection Act and approved by the compet- ent authority (Thuringian State Office for Consumer Protection, reg. no. 22-2684-04-04-102/13 and reg. no. 04-004/1).

**Culture of macrophages for studying *C. burnetii* invasion and replication kinetics.** Invasion and replication of *C. burnetii* strains NMI and NMII were studied with macrophages (5 x 105 cells/well) cultivated in polyostyrene tubes (Greiner). MDM cultures were inoculated at an MOI of 100 by addition of bacteria to IMDM culture medium 3 for 1 h. Preceding experiments had revealed that increasing the MOI to 200 and/or the inoculation time to 2 h did not result in significantly higher bacterial numbers 7 days after inoculation. After 1 h, macrophages were washed 3
times with 0.89% NaCl solution and replenished with IMDM culture medium 3. Triplicate cell cultures were harvested at different time points to monitor C. burnetii invasion and replication efficacy. Cells were lysed by three freeze/thaw cycles and extensive vortexing (5 min). For quantification by PCR, cell suspension was additionally inactivated by boiling (95°C, 20 min). Replication rates were calculated by quantification of the 16S gene by absolute quantitative real-time PCR (37). C_t values (where C_T is threshold cycle) of technical duplicates varied by less than 0.51 and were used to calculate genome equivalents (GE) considering values obtained with an entrained/icd-harboring plasmid standard. To enumerate viable bacteria, infected macrophages were lysed by a 3-fold thaw-freeze cycle. From the resulting suspension, the median tissue culture infective doses (TCID₅₀) were defined by endpoint titration on BGM cells (38). In a 4-fold approach, confluent BGM cells in a microtiter plate (Nunc F96 MicroWell; Nunc, Wiesbaden, Germany) were inoculated with a log₂ dilution series of the sample (dilution with cell culture medium, consisting of 300 ml Eagle’s minimum essential medium with Earle’s salts supplemented with NaHCO₃ [0.055 g/liter], 5 ml l-glutamine, 5 ml Vitamin 100X solution, and 50 ml FCS [all from Biochrom, Berlin, Germany]). After sealing the wells with adhesive film (Nunc) and centrifugation at 2,400 x g for 60 min at 30°C, the plate was incubated at 37°C. On day 8, wells containing C. burnetii-infected BGM cells were identified by phase-contrast microscopy (Nikon Eclipse TS100-F). C. burnetii-specific vacuoles were considered, and each well having at least one C. burnetii vacuole was counted as positive. The TCID₅₀ was calculated according to the method of Spearman and Kärber (39).

**Cultivation of macrophages for RNA isolation.** Macrophages (2 x 10⁶ cells/well) were cultured in 6-well culture plates and inoculated with C. burnetii strains NMI or NMII as described above. As controls, cells were challenged with either a heat-killed suspension of NMI (95°C, 10 min). A stock solution of 10³ C. burnetii/ml was prepared in Eagle’s minimum essential medium supplemented with 0.5% BSA, and 100 µl of this solution was added to each well. After 48 h, the supernatant was collected and stored at -80°C until RNA extraction was performed. RNA was isolated with the RNeasy minikit (Qiagen) according to the instructions of the manufacturer. To avoid DNA contamination, RNA was purified with the RNase-free Dnase kit (Qiagen).

**RT and cytokine-specific real-time PCR.** Equal amounts of RNA from each sample were reverse transcribed into cDNA. First, each sample was denatured for 5 min at 65°C and directly cooled on ice. After that, samples were incubated for 60 min at 37°C using 4 U reverse transcriptase (Qiagen) and the appropriate buffer containing 0.5 mM deoxynucleoside triphosphates (dNTPs; Qiagen), 1 µM random primer, and 20 U RNase inhibitor (Promega, Mannheim, Germany). The reaction was terminated by heating to 93°C for 5 min. For excluding the presence of DNA, reverse transcription (RT) controls that were composed of sample and mastermix without reverse transcriptase were included and gave yield to negative results throughout. Thereafter, the levels of relative gene expression of different host-specific cytokines in comparison to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene were determined by quantitative real-time SYBR green-based (Applied Biosystems, Waltham, MA, USA) PCR using an ABI Prism7500 (Applied Biosystems). All primers (Table 1) were exon-intron spanning and run at an annealing temperature of 60°C. The PCR profile was as follows: denaturation (15 s, 95°C), annealing (1 min, 60°C; 39 cycles), and melting step (15 s, 60°C). PCR products quantified over cycle 38 are not valid. Relative gene expression levels were calculated by using the relative expression software REST (40).

**Cultivation of macrophages for flow cytometry analysis.** Macrophages (2 x 10⁶ cells/well, 6-well plates) were inoculated with C. burnetii (MOI, 100) for 1 h. After 24 h of incubation, cells were detached by incubation with Accutase (PAA Laboratories, Germany), transferred to microtiter plates (V-shape; Greiner), and pelleted by centrifugation (400 x g, 4 min and 4°C). For detection of surface proteins (CD40, CD80, CD86, major histocompatibility complex class I [MHC-I] and MHC-II, CD11b [CR3], CD61 [α₂β₁]), cells were incubated with 50 µl diluted primary antibody for 20 min (Table 2). After washing in PBS–0.5% FCS, cells were fixed with paraformaldehyde (4% in PBS, 4°C, 24 h). Thereafter, cells were washed and incubated with secondary antibody (mouse anti-C. burnetii IgG, anti-mouse IgG2a-APC, anti-mouse IgG2b-PE; Southern Biotech, USA) diluted 1:1,000 in PBS for 20 min. Finally, cells were washed again and analyzed with BD FACSCTantoII (Becton-Dickinson, Heidelberg, Germany). Data were analyzed with BD FACSDIVA software (version 6).

### Interleukin-1β ELISA.

For interleukin-1β enzyme-linked immunosorbent assay (ELISA), infected macrophages (2 x 10⁶ cells/well; MOI, 100) were incubated for 24 h in 6-well plates. Supernatant was collected and treated with UV light. Pretests had shown that this procedure inactivated C. burnetii (no vacuole formation suggesting C. burnetii replication after four passages on BGM cells). Samples were concentrated by using Amicon Ultra 4 columns (Millipore, Darmstadt, Germany). Microtiter plates (MaxiSorb; Nunc-Thermo Fisher Scientific, Braunschweig, Germany) were coated with capture antibody (MCA1658; AbD Serotec, Puchheim, Germany; 100 ng/well in coating buffer [15 mM Na₂CO₃, 34 mM NaHCO₃, and 500 ml distilled water]) overnight at 4°C. After washing the wells twice with washing buffer (PBS, 0.05% Tween 20), samples and a dilution series (400 ng/ml through 1:10) of recombinant interleukin-1β standard (PBP008; AbD Serotec) were added for 1 h. After another washing step with blocking buffer (PBS, 0.05% bovine serum albumin [BSA]), primary antibody (AHP423, 1:1,000 in blocking buffer; AbD Serotec) was added and incubated for 1 h. Following one washing step, wells were incubated with horseradish peroxidase-coupled secondary antibody (STAR124P; AbD Serotec) and 1:50,000 diluted secondary antibody (anti-mouse IgG1-APC, anti-mouse IgG2a-APC, anti-mouse IgG2b-PE; Southern Biotech, USA) diluted 1:1,000 in PBS for 20 min. Finally, cells were washed again and analyzed with BD FACSCTantoII (Becton-Dickinson, Heidelberg, Germany). Data were analyzed with BD FACSDIVA software (version 6).

**Western blot analysis.** MDM (2 x 10⁶ cells/well, 6-well plates) were inoculated with C. burnetii (MOI, 100) for 24 h. Total cellular protein was sampled by lysis cells with 500 µl Laemmli buffer (0.5 M Tris–HCl, 87% glycerol, 10% SDS, 4% β-mercaptoethanol, 0.05% bromophenol blue) and boiling at 100°C for 10 min. Protein concentration was measured by the Bradford method (41). Equivalent protein amounts from each sample and a positive control (recombinant bovine interleukin-1β protein) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, Bio-Rad). Blots were incubated with blocking buffer (5% milk, 0.1% Tween 20) for 1 h. Diaminobenzidine (DAKO, Hamburg, Germany) was used as a substrate, and the reaction was stopped by addition of 100 µl of 1 N H₂SO₄.

### TABLE 1 Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>F, GGC ATA CTC ACT CTT CTA CCT TCG A R, TCG TAC CAG GAA ATG AGC TGG TAC AC</td>
</tr>
<tr>
<td>IL-1-β</td>
<td>F, ACC TGA ACC CATCAA CAA GAT A G R, TAG GGT CAT CAG CCT CAA ATA ACA</td>
</tr>
<tr>
<td>IL-10</td>
<td>F, GTG ATG CAA CAG GCT GAG AA R, TGC TCT TGT TTT CGG AGG GCA</td>
</tr>
<tr>
<td>INF-γ</td>
<td>F, TTC TTG AAC GGC AGC TCT GAG R, TGG CGA CAG GTC ATT CAT CA</td>
</tr>
<tr>
<td>TGF-β</td>
<td>F, GGC CCT GCC CCT ACA TCT G G R, CGG GTT GTG CTG GTT GTA CA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F, TCT TCT CAA GCC TCA AGT AAC AAG T R, CCA TGA GGG CAT TGG CAT AC</td>
</tr>
</tbody>
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* F, forward; R, reverse.
by SDS-PAGE (4 to 12%) and blotted onto a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific). Nonspecific binding sites were blocked by incubation with skimmed milk. Tris-buffered saline with Tween 20 (100 mM NaCl, 100 mM Tris, 5 mM MgCl₂·6 H₂O, 0.05% Tween 20) was used for washing steps. The membrane was incubated overnight with primary antibody (AHP 423, 1:1,000 in PBS-Tween). After washing, an alkaline phosphatase-conjugated secondary antibody (anti-mouse-IgG1-AP, 1:2,000 in PBS-Tween) was applied for 1.5 h. Protein was visualized by using a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride detection system.

Statistical analysis. Statistical comparisons were conducted using the t test or U test with statistic software XLSTAT. Real-time PCR data were analyzed by a randomization test with pairwise reallocation (software REST [40]).

RESULTS

C. burnetii internalization and replication in bovine macrophages. Cultured MDM expressed the C. burnetii receptors CR3 and α₃β₁ integrin (42) on their surface (Fig. 1A). The same was true for alveolar macrophages, which surface expressed C. burnetii receptors when freshly isolated from BAL fluid.

Macrophages from both sources internalized C. burnetii strains NMI and NMII in general but with different efficiencies. The numbers of C. burnetii genome equivalents (GE) determined varied considerably between biological replicates, i.e., different cultures of primary cells. However, the invasion efficacy of NMI into bovine MDM was significantly higher, with approximately 10% of the C. burnetii bacteria being cell bound 1 day postinoculation (dpi), while only a small portion of the NMII inoculum was cell associated at that point in time (Fig. 1B). Different from what was seen with MDM, similar low mean percentages of the inoculated NMI and NMII bacteria were associated with alveolar macrophage cells within 1 dpi.

Despite marked biological variation between cultures, bovine MDM and alveolar macrophages generally permitted C. burnetii replication. After an initial decline in absolute GE numbers from day 1 to day 3 of inoculation in MDM (NMI) and alveolar macrophages (NMI, NMII), bacterial replication started mostly at 3 dpi and did not cease until day 14. However, no explicit replication was evident in NMII-infected bovine MDM (Fig. 2). When applying a retitration method on BGM cells to more closely monitor the number of viable C. burnetii cells in bovine MDM cultures, NMI and NMII both showed an increase in numbers by about 2 orders of magnitude within 14 dpi (Fig. 3A and B). While the numbers of NMI had increased 243- ± 219-fold (mean values for three independent experiments and respective SD), the numbers of NMII increased 56- ± 35-fold, but the values were not statistically significantly different (P = 0.2). The ability of C. burnetii to establish an intracellular replicative niche in bovine MDM

![FIG 1](http://iai.asm.org)  
Expression of Coxiella burnetii-specific receptors and internalization of C. burnetii into bovine macrophages. (A) Uninfected bovine MDM (top) and alveolar macrophages (bottom) were analyzed by FACS for expression of CR3 and α₃β₁ on their surface. Gray-shaded curves depict detection of the respective antigens, and black lines represent the secondary antibody control (representative results of two technical replicates in 2 independent experiments each). (B) The numbers of cell-associated C. burnetii bacteria at 1 dpi were calculated based on the number of genome equivalents (GE) quantified by icd-specific real-time PCR and expressed as percentage of GE in the inoculum (box plot of twice-performed technical determinations in 3 independent experiments; **, P ≤ 0.01; n. s., not significantly different).
was further corroborated by the formation of parasitophorous vacuoles (Fig. 3C), which were indistinguishable in shape and size between the two strains (data not shown). Small PVs were located mostly in groups close to the cellular membrane and fused in later stages of the replication cycle to larger PVs. Coxiella-infected macrophages retained their viability and integrity over the entire time course of the experiments as demonstrated by light microscopy and trypan blue exclusion tests at days 1, 7, and 14 after inoculation.

**Bovine macrophages rapidly responded to *C. burnetii* by differential transcription of cytokine genes.** MDM responded to *C. burnetii* by transcription of different cytokine genes as early as 3 hpi (Fig. 4A). NMI significantly increased the amounts of mRNA specific for some Th1-related cytokines, such as IL-1β, IL-12, and tumor necrosis factor alpha (TNF-α), but not for gamma interferon (IFN-γ). Induction of Th2-related cytokines was less prominent (IL-10), or the amounts of specific mRNA were not different from those of uninoculated MDM cultures (transforming growth factor beta [TGF-β]). Inoculation with *E. coli* LPS (6 μg/ml) and a heat-inactivated NMI suspension were used as stimulation controls. Both induced a significant up-regulation of proinflammatory cytokine transcription 3 hpi (Fig. 4A), whereas inoculation with a heat-killed suspension of NMI led to MDM responses indistinguishable from those obtained with inoculation with viable NMI. Induction of cytokine expression correlated with the LPS phase, as the MDM response to NMII inoculation was not different from the medium control or significantly lower than the response to NMI. In contrast to MDM,

**FIG 2** Replication of *C. burnetii* in bovine MDM (A) and in alveolar macrophages (B). Cells were inoculated with *C. burnetii* strains NMI or NMII (MOI of 100 for 1 h), and *C. burnetii*-specific genome equivalents (GE) were quantified in duplicate at different time points by *icd*-specific real-time PCR and calculated by use of a standard curve. Kinetic of *C. burnetii* replication was monitored in 3 independent experiments depicted by different symbols within the curves.
alveolar macrophages responded to *C. burnetii* in a more restrained manner (Fig. 4B). Remarkably, viable NMII induced a 4.5-fold-more-pronounced increase in the IL-1β mRNA level than that induced by viable NMI, while a heat-killed NMI suspension led to marked transcription of IL-1β and IL-12. NMII only slightly induced transcription of IL-10 and IFN-γ, whereas *C. burnetii* did not affect TGF-β transcription in alveolar macrophages.

The cytokine response of bovine MDM to prolonged exposure to *C. burnetii* was transient. Increased transcription of cytokines rapidly waned during *C. burnetii* infection of MDM. While NMI induced large amounts of mRNA specific for IL-1β, IL-12, and TNF-α at 3 hpi, the amounts decreased to near-background values (medium control) with increasing intracellular *C. burnetii* numbers following bacterial growth (Fig. 5). The transient character of the cytokine response was independent of bacterial replication, as inoculation with a heat-killed NMI suspension yielded similar results. These findings contrast with results from two control experiments showing that *E. coli* LPS as well as *C. psittaci* inoculation led to a further increase of at least IL-12 transcription from 3 or 4 h to 1 day after addition of the stimulus (Fig. 6).

*C. burnetii* prevented IL-1β translation and release in bovine MDM culture. In contrast to other proinflammatory cytokines, IL-1β is expressed as a precursor protein and requires an enzyme-controlled cleavage process for formation of the bioactive cytokine. Quantitative analysis by ELISA revealed that after 24 h of incubation, IL-1β protein was only detectable in supernatants of LPS-stimulated MDM cultures (Fig. 7A). Inoculation of MDM cultures with NM variants did not result in detectable release of IL-1β beyond that of an uninfected control culture. These findings were corroborated by Western blotting showing that stimulation with *E. coli* LPS led to expression of precursor protein and cleavage products of different sizes while inoculation with viable and heat-killed *C. burnetii* suspensions did not (Fig. 7B).

*C. burnetii* only marginally affected macrophage maturation. *E. coli* LPS and the heat-killed NMI suspension intensified MDM maturation triggered by plastic adherence as deduced from an increase in CD40 and CD80 expression, while CD86 and MHC expression were only slightly affected by *E. coli* LPS stimulation (Fig. 8A). In contrast, cell maturation was barely affected by viable NMII. Only viable NMI induced a weak expression of CD40 (*P* = 0.0001) and CD80 compared to the untreated control. Although alveolar macrophages responded less vigorously to stimulation, the overall reaction pattern to *C. burnetii* was similar to that of MDM (Fig. 8B).

**DISCUSSION**

The currently limited understanding of the infection route of *C. burnetii* in ruminants has direct implications for designing strategies to control the epizootic pathogen and to protect humans from a zoonotic disease. Macrophages are accepted as the major host cell for *C. burnetii* infection, and rodent studies reported high numbers of *C. burnetii* in alveolar macrophages (43). Infection models for studying host-pathogen interactions have already been established with human, mice, and monkey macrophage cultures (18, 44, 45). Here, we present the first in vitro study demonstrating that primary macrophages from cattle can be utilized in an infection model for investigating the lifestyle of *C. burnetii* in the ruminant host.

Internalization of *Coxiella* into macrophages occurs by phagocytosis and is mediated by αvβ3 integrin for virulent phase I particles, whereas avirulent phase II particles additionally require the CR3 receptor (42). We found that bovine MDM and alveolar macrophages express high numbers of both receptors on their surface. Internalization of virulent NMI was more efficient than that of the avirulent strain NMII. Interestingly, this prominent difference between the strains was observed only in bovine MDM cultures but not when alveolar macrophage cultures were inoculated in which equally low numbers of *C. burnetii* bacteria expressing either LPS phase became cell bound. As opposed to what is seen with bovine macrophages, phase I particles are only poorly internalized by human monocytes, whereas phase II particles are better ingested (46). It remains to be determined whether these apparent species differences can be traced back to quantitative differences in the expression of αvβ3, CR3, or other surface molecules like, e.g., Toll-like receptor 4 (TLR4) by phagocyte subsets.

Despite quantitative differences in internalization, phase I and phase II strains replicated with similar kinetics in bovine MDM. NMI and NMII replication became particularly apparent when viable bacteria were retitrated in an independent cell system commonly deployed for *C. burnetii* isolation for diagnostic purposes. Irrespective of LPS phase, *C. burnetii* initiated proliferation once
bacteria were intracellular and apparently had escaped from the antibacterial activities of the phagocytes as in human MDM (47). *C. burnetii* was reported to productively proliferate in human alveolar macrophages as well (18). Due to the limited number of bovine alveolar macrophages available for this study and the laborious replating assay, only quantitation of genome equivalents (GE) was applied to assess *C. burnetii* replication in this cell type (37). While NMI-inoculated alveolar macrophages showed a clear increase in GE numbers from day 1 to day 14 of culture, GE numbers in NMII-inoculated cultures increased slightly between days 3 and 7 (P = 0.041). Counting the infected cells in a replating assay has the big advantage of allowing the quantification of viable cells, whereas RT-PCR may also quantify *C. burnetii* particles that are deficient in infectivity or replication. In order to permit massive parallel testing of a large number of samples, the assay read-out was by phase-contrast microscopy, which may have impaired the sensitivity of this approach. The characteristics of the methods likely explain the discrepancies in absolute numbers of genome equivalents and 50% tissue culture infective doses observed in this study. It remains to be determined, by titration on susceptible cells, whether bovine alveolar macrophages are capable of effectively limiting growth of phase II organisms or whether the abundance of GE from nonviable bacteria had masked the detection of replication.

Macrophages are one of the first barriers of the innate immune system against pathogens. During the early phase of infection, *C.

**Figure 4** Influence of *C. burnetii* on cytokine expression 3 h after inoculation of bovine MDM (A) and alveolar macrophage (B) cultures. Cells were inoculated with *C. burnetii* strain NMI or NMII (MOI of 100 for 1 h). Addition of a heat-killed suspension of NMI (NMI_HI) at an equivalent bacterial cell-to-macrophage ratio or *E. coli* LPS (6 μg/ml) served as a control. Amounts of cytokine mRNA were normalized to GAPDH mRNA and determined relative to the cell control (set to 10^10^). Results are depicted as mean values with their respective SD. A randomization test with a pairwise reallocation was used to compare ∆∆C_T values from 3 independent experiments (*, P ≤ 0.05; **, P ≤ 0.01; significantly different from cell control results).
Burnetii induced a rapid expression of Th1-related cytokines like IL-1β, IL-12, and TGF-β in bovine macrophages. The same response pattern was observed in murine macrophages with strong expression of IL-1β, IL-12, and TNF-α at 3 hpi (17). Cytokine expression in bovine macrophages differed after inoculation with strains possessing different LPS phase types, with NMI inducing more-pronounced responses than NMII under the experimental conditions applied, i.e., exposure to C. burnetii particles at an MOI of 100. Phase-dependent induction of inflammatory host cell responses also occurs in human dendritic cells, but full-length LPS of virulent C. burnetii appears to mask TLR ligands from innate immune recognition in this setting (48). If the target cell does become activated by C. burnetii, TLR2 recognition of the lipid A moiety of LPS (49) is implicated in the induction of proinflammatory responses (50). LPS phases in Coxiella vary in the carbohydrate composition of the O chain. Lack of certain sugars results in a shorter chain length and loss of the TLR ligand masking function. In contrast, differences between the two C. burnetii phase types in their lipid A structure have not been reported (51). Other microbe-associated molecular patterns of C. burnetii, the binding of which to bovine macrophages is not efficiently prevented by phase I LPS, may therefore account for the differences observed.

C. burnetii and other intracellular bacteria induce proinflammatory responses by host cell attachment independent of the internalization process or its efficiency (52–54). Particularly, TNF expression was shown to be induced by binding of C. burnetii to

**FIG 5** Influence of C. burnetii on cytokine expression by bovine MDM over time. Cells were inoculated with C. burnetii strain NMI or NMII (MOI of 100 for 1 h) or with a heat-killed suspension of NMI (NMI_HI) at an equivalent bacterial cell-to-macrophage ratio. Amounts of mRNA of different cytokines were normalized to GAPDH mRNA and determined relative to the cell control (set to 10^0) at different time points. A randomization test with a pairwise reallocation was used to compare ∆∆C_T values from 3 independent experiments. Results are depicted as mean values with their respective SD (*, P ≤ 0.05; **, P ≤ 0.01).

**FIG 6** IL-12 expression by bovine MDM upon exposure to E. coli LPS or infection with Chlamydia psittaci for 3 or 4 and 24 h. Amounts of IL-12-specific mRNA were normalized to GAPDH mRNA and determined relative to cell control (set to 10^0) at different time points. Results are depicted as mean values ± SD for 3 independent experiments. Statistical differences are indicated (*, P = 0.05; **, P = 0.01).
FIG 7 C. burnetii induced IL-1β activation in bovine MDM. (A) Cells were inoculated with C. burnetii strain NMI or NMII (MOI of 100 for 1 h), with a heat-killed suspension of NMI (NMI_HI) at an equivalent bacterial cell-to-macrophage ratio or stimulated with E. coli LPS or mock inoculated with NaCl solution (CC). Supernatants were collected 24 h later. The amount of mature IL-1β was analyzed by ELISA. Results from 3 independent experiments are depicted as mean values ± SD, and statistical differences are indicated (**, P ≤ 0.01). (B) Total protein was isolated from infected or stimulated MDM at 24 hpi. Equal protein amounts from each sample were separated on a 4 to 12% SDS-polyacrylamide gel and immunolabeled for IL-1β. Bovine recombinant IL-1β served as a control for the mature protein.

the cell (52). A heat-killed suspension of NMI induced a cytokine expression pattern in bovine macrophages similar to that induced by viable NMI. Heat-killed NMI was detected intracellularly by staining inoculated bovine MDM with anti-NMI antibodies (data not shown), proving that the cells recognize nonviable NMI particles as to-be-phagocytosed material. C. burnetii uptake is not solely an actively steered process, and surface-located factors, such as outer membrane protein (Omp) A or heat shock proteins (Hsp), could be implicated in the regulation process. OmpA is a highly conserved outer membrane protein among Gram-negative bacteria that has an important role in pathogenesis, including adherence, invasion, and activation of host responses (55) through binding to TLR2 (56, 57). Coxiella-expressed OmpA triggers an adhesion-mediated internalization of bacteria by nonphagocytic cells, e.g., epithelial cell, but not by macrophages (58). Heat-stable Hsps govern the early phase of Legionella infection in macrophages and induce a rapid increase of IL-1β mRNA by a protein kinase C (PKC)-dependent signaling pathway (59). Hsp70 is located on the surface of C. burnetii and homologous to Hsps of Legionella (60). C. burnetii of the two LPS phase types bound to and invaded bovine alveolar macrophages equally well, followed by minor differences in the accompanying host cell response. In bovine MDM, strikingly different invasion rates of NMI and NMII mirrored quantitative variations in proinflammatory cytokine expression. The early host cell activation in bovine macrophages thus seems to result from bacterial cell adhesion and/or internalization and occurs independently of bacterial metabolic activity.

While C. burnetii productively replicated in bovine MDM, enhanced expression of certain cytokines rapidly declined during later stages of infection to the levels of uninfected cell controls (7 dpi). In order to determine if the observed effect is specific for infection with C. burnetii, we exemplarily compared the temporal response pattern of IL-12 transcription in bovine MDM cultures to that in E. coli LPS and another intracellular pathogen, C. psittaci. C. psittaci has a broad host range, including cattle (61), and replicated efficiently in the bovine MDM cultures (K. Hillarius and A. Berndt, data not shown). Contrary to NMI infection, both treatments induced increasing IL-12 expression during the first 24 h, implying that C. burnetii specifically controls the host response in bovine macrophages to allow replication without significant inflammatory cytokine production. Probably by different molecular mechanisms but similar to that of human DCs (48), this immune evasion strategy may allow C. burnetii to productively infect and replicate in an immunocompetent host.

IL-1β is one of the most potent endogenous pyrogens and instrumental for the host defense against pathogens (62). The synthesis of this protein is strictly regulated. A precursor of IL-1β is cleaved by caspase 1 to mature and highly active IL-1β, which then is secreted into the extracellular space (63, 64). Bovine MDM responded to C. burnetii infection (3 hpi) with profound transcription of the IL-1β gene. However, in contrast to E. coli LPS stimulation, we found neither mature IL-1β in culture supernatants of these macrophages nor the precursor in their whole-cell lysates after 24 h. A heat-killed suspension of NMI also prevented IL-1β translation, implying that the conversion of gene transcription activation into protein-encoded information was blocked by processes not requiring active bacterial metabolism. Different from monocytes, caspase 1 is not constitutively activated in macrophages, which require free ATP in addition to, e.g., stimulation by LPS, to activate the enzyme (65, 66). Splicing forms of IL-1β were detectable in LPS-stimulated bovine MDM, indicating that ATP was available in the system and probably released from cells that underwent cell death during the isolation or cultivation process of these primary cell cultures. Intracellular bacteria, such as Chlamydia or Mycobacterium, inhibit the influx of ATP to avoid the maturation and secretion of the IL-1β protein (67, 68). It needs to be elucidated whether the response of bovine macrophages is restricted by C. burnetii at the level of posttranscriptional modification and whether this also applies to other proinflammatory cytokines.

The generation of an adaptive immune response emanates from T cell activation by antigen-presenting cells and involves specific surface proteins (MHC molecules) as well as costimulatory molecules (CD40, CD80, CD86) (69). Tissue-resident macrophages normally express only few such costimulatory molecules on their surfaces. Only after ingestion of foreign material may cells change their surface phenotype, downregulate adhesion molecules, migrate to draining lymph nodes, and convert into effective antigen-presenting cells by increasing the surface expression of...
molecules essential for the interaction with naive T cells. Interestingly, neither infection with the virulent strain NMI nor infection with the avirulent strain NMII led to a substantial maturation of bovine macrophages in our study. While formalin-killed NMI failed to induce cell maturation in human DCs (48), a heat-killed NMI suspension induced an increase of CD40 and CD80 expression in bovine MDM. Further studies are needed to determine whether the maturation of bovine antigen-presenting cells induced by preformed Coxiella factors is counteracted by components secreted by actively replicating bacteria.

The depression of macrophage maturation and the short duration of proinflammatory cytokine expression (IL-1β, IL-12, TNF-α) during the first 3 hpi demonstrates that C. burnetii induced a combination of M1 and M2 polarization of bovine macrophages, i.e., an atypical M2 polarization resulting in M2-typical properties dominating over M1-typical properties as reported for human macrophages (70). It is well established that intracellular bacteria resist antimicrobial effectors to survive in the host cell (71). During acute Q fever infection in humans, an atypical M2 polarization of macrophages promotes long-term survival of C. burnetii with a slow replication rate, whereas M1-polarized monocytes control the infection. During chronic Q fever, monocytes

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**FIG 8** Surface marker expression by bovine MDM (A) and alveolar macrophages (B) in response to *C. burnetii* infection. Cells were inoculated with *C. burnetii* strain NMI or NMII (MOI of 100 for 1 h) or with a heat-killed suspension of NMI (NMI_HI) at an equivalent bacterial cell to macrophage ratio or stimulated with *E. coli* LPS. Twenty-four hours later, cells were immunolabeled for the surface markers CD40, CD80, CD86, and MHC-I and -II. Results of 3 to 8 independent experiments are shown as mean values ± SD, and statistical differences are indicated (*, \( P < 0.05; **, \( P < 0.01; significantly different from cell control results).
and macrophages exhibit an M2 polarization with an intense replication of C. burnetii (72).

In conclusion, bovine macrophages may serve (i) as a vehicle for C. burnetii dissemination through the organism to the intended host cells for replication (e.g., epithelial cells in placenta, udder, or gut) and (ii) as a niche for bacterial long-term survival, given the long life span of macrophages (73) and the comparably low replication efficiency of C. burnetii in bovine macrophages. Although cattle are not the main source of human infection, the bovine macrophage model established and characterized here can be used to study host-pathogen interactions in a reservoir host at the cellular level, allowing to characterize replication and the pathogen-induced host cell response to different C. burnetii strains, e.g., the species-specific adaptations of the highly prevalent C. burnetii genotype ST 20 recently identified in bovine milk in the United States (74).

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