Murine Alveolar Macrophages Are Highly Susceptible to Replication of *Coxiella burnetii* Phase II *In Vitro*


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*Coxiella burnetii* is a Gram-negative bacterium that causes Q fever in humans. Q fever is an atypical pneumonia transmitted through inhalation of contaminated aerosols. In mammalian lungs, *C. burnetii* infects and replicates in several cell types, including alveolar macrophages (AMs). The innate immunity and signaling pathways operating during infection are still poorly understood, in part because of the lack of relevant host cell models for infection *in vitro*. In the study described here, we investigated and characterized the infection of primary murine AMs by *C. burnetii* phase II *in vitro*. Our data reveal that AMs show a pronounced M2 polarization and are highly permissive to *C. burnetii* multiplication *in vitro*. Murine AMs present an increased susceptibility to infection in comparison to primary bone marrow-derived macrophages. AMs support more than 2 logs of bacterial replication during 12 days of infection in culture, similar to highly susceptible host cells, such as Vero and THP-1 cells. As a proof of principle that AMs are useful for investigation of *C. burnetii* replication, we performed experiments with AMs from Nos2−/− or Ifng−/− mice. In the absence of gamma interferon and nitric oxide synthase 2 (NOS2), AMs were significantly more permissive than wild-type cells. In contrast, AMs from Il4−/− mice were more restrictive to *C. burnetii* replication, supporting the importance of M2 polarization for the permissiveness of AMs to *C. burnetii* replication. Collectively, our data account for understanding the high susceptibility of alveolar macrophages to bacterial replication and support the use of AMs as a relevant model of *C. burnetii* growth in primary macrophages.

The intracellular bacterial pathogen *Coxiella burnetii* is the causative agent of the zoonotic infection termed Q fever. Human infection with *C. burnetii* can lead to asymptomatic seroconversion or symptomatic Q fever, which often presents as an acute febrile illness. The nonspecific symptoms associated with acute infection indicate that it is likely significantly underdiagnosed. Acute infection is often self-limited, but in a minority of cases it can progress to a serious chronic infection that predominantly manifests as life-threatening endocarditis. Importantly, recent epidemiological data demonstrated the significant long-term health impact of Q fever reporting that more than one in three patients continued to suffer from an impaired health status 24 months postdiagnosis (1).

Humans can become infected through the inhalation of contaminated aerosols, often from close contact with infected ruminants. Within the alveolar space, *C. burnetii* can infect a variety of cell types, including alveolar macrophages (AMs) (2). Essential to the capacity of *C. burnetii* to cause disease is the ability of the pathogen to replicate inside host cells within a unique lysosome-derived vacuole. The infectious, or small-cell variant, form of the bacterium enters the host cell and is passively trafficked through the endocytic pathway before reaching the hydrolytic and acidic confines of the lysosome. These conditions trigger the metabolic activation of *C. burnetii* and stimulate the conversion to a large-cell, replicative variant (3). This environment also triggers the active bacteria to assemble the Dot/Icm type IV secretion system that facilitates the translocation of over 130 effector proteins into the host cytoplasm (4). Collectively, the action of these effectors modulates the *Coxiella*-containing vacuole (CCV) to provide an environment conducive to replication (5). This intracellular life cycle holds true for both virulent (phase I) *C. burnetii* and avirulent (phase II) *C. burnetii* bacteria that have a lipopolysaccharide structure altered through genetic mutations that occur during serial passage in a nonimmunocompetent host (6).

It is clear that while development of the CCV is similar in many cell types, some cells, including primary peritoneal macrophages and bone marrow-derived macrophages (BMDMs), are intrinsically more restrictive to the intracellular replication of *C. burnetii* (7, 8). Furthermore, BMDMs from different inbred mouse strains vary in their ability to restrict *C. burnetii* infection, with A/J and BALB/c mice being more susceptible to *C. burnetii* phase II than many other mouse strains, including the C57BL/6 strain (9). The mechanisms through which different genetic backgrounds and cell types can control *C. burnetii* intracellular growth remain an area of interesting scientific pursuit.

AMs have long been considered the primary site of *C. burnetii* infection (2), and AMs from monkeys and humans have recently been used to explore the host-pathogen interactions that take place during *C. burnetii* infection. Cynomolgus monkey AMs were used to demonstrate the potent antiapoptotic activity asso-
associated with *C. burnetii* infection, although the replication dynamics of *C. burnetii* in this cellular model were not explored (10). More recently, human AMs, extracted from postmortem lung tissue samples, were shown to support the replication of different pathotypes of *C. burnetii* (11). In addition, this study demonstrated that both virulent (phase I) and avirulent (phase II) *C. burnetii* bacteria are able to infect human AMs. Interestingly, it was also observed that, within human AMs, *C. burnetii* more frequently forms multiple smaller CCVs rather than the large fusogenic vacuole observed in other cellular models of infection (11). Within a murine model of infection, it has been demonstrated that AMs are susceptible to *C. burnetii* infection (12), and an early study demonstrated that nitric oxide (NO) is produced by murine AMs in response to infection (13).

In the study described here, we investigated and characterized the infection of primary murine AMs by *C. burnetii* phase II in vitro. Our data reveal that AMs are permissive to *C. burnetii* multiplication. The AMs showed a pronounced M2 polarization and showed an increased susceptibility to infection in comparison to murine BMDMs. Finally, we investigated the contribution of signaling molecules to the susceptibility of AMs to *C. burnetii*, demonstrating the utility of AMs for investigation of the host restriction of *C. burnetii* infection in relevant host cells.

**MATERIALS AND METHODS**

Preparation of *C. burnetii* for infection. *Coxiella burnetii* phase II Nine Mile strain (RSA439) bacteria were prepared from infected Vero cell monolayers as previously described (14). Confluent cultures of Vero cells were irradiated with 10 Gy 60Co to block cell division and maintained at 37°C in 5% CO2 in Dulbecco modified Eagle medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco) and 1 mM l-glutamine (Sigma-Aldrich). Infection was carried out, and the cells were maintained under the above-described conditions for an additional 6 days. For the preparation of the *Coxiella* inoculum, infected Vero cells were lysed by homogenization in sterile distilled water. Cell lysates were clarified by centrifugation at 1,810 × g for 10 min at 4°C. The supernatant was extracted and centrifuged at 12,857 × g for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in RPMI 1640 medium with 10% FBS. Bacterial aliquots were kept at −80°C. Prior to infection, aliquots were thawed at 37°C and subjected to sonication in 4°C for 10 min. For assays with THP-1 cells, differentiation into macrophage-like cells was mediated by the addition of 200 nM phorbol 12-myristate 13-acetate (PMA) to the culture overnight as described previously (15). Briefly, bone marrow-derived macrophages. BMDMs were obtained and differentiated according to the protocol described previously (15). Briefly, bone marrow cells were harvested from femurs and differentiated with RPMI 1640 medium (Sigma-Aldrich) containing 20% FBS (Invitrogen) and 30% L-929 cell conditioned medium (LCCM) at 37°C in 5% CO2 for up to 12 days. After 6 days of infection, cells were supplemented with an additional 500 µl of RPMI 1640 medium with 10% FBS and 5% LCCM for 6 postinfection.

**C. burnetii infection of cells.** Frozen stocks of *C. burnetii* were thawed at 37°C and treated in an ultrasound bath for 3 min to dissolve bacterial clumps. Tissue culture medium was aspirated from the cells and replaced with fresh medium before infection. Bacteria were homogenized with a pipette and applied directly to the cells at a multiplicity of infection (MOI) of 3 (except for the experiment whose results are presented in Fig. 1A, in which an MOI of 100 was used). The plates were centrifuged at 200 × g for 5 min at 25°C and placed at 37°C in 5% CO2. After 4 h, the culture medium was aspirated, the wells were washed with warm sterile PBS, and fresh culture medium was added. As an internal experimental control of *C. burnetii* replication, infected cells were treated with 20 µg/ml rifampin (RIF) to inhibit *C. burnetii* protein translation (16).

**Fluorescence microscopy.** AMs from C57BL/6 mice were seeded at a density of 105 cells per well in a 24-well plate containing 13-mm glass coverslips. Cells were infected with wild-type *C. burnetii* bacteria or *C. burnetii* bacteria expressing mCherry at an MOI of 3 as previously described (17). After 4 h, 3 days, 6 days, 9 days, or 12 days of infection, cells were fixed with 3.5% paraformaldehyde containing 7.5% sucrose for 20 min and then washed twice with room temperature PBS. For the assay with wild-type *C. burnetii* bacteria, the cells were then permeabilized for 2 min with ice-cold 100% methanol, followed by two more washes with PBS. Cellular autofluorescence was removed by incubation with 0.1 M glycine in PBS for 10 min, and then the samples were blocked for 30 min in PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich) and 5% goat serum at room temperature. The cells were incubated with rabbit anti-*C. burnetii* (1:100) and rat anti-LAMP-1 (1:2) primary antibodies diluted in 1% BSA in PBS for 1 h, washed twice with room temperature PBS, and then incubated for 30 min in 1% BSA in PBS containing Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Invitrogen) at a concentration of 1:2,000. After three washes with room temperature PBS, cells were mounted with Prolong Gold antifade reagent containing DAPI (4',6'-diamidino-2-phenylindole dilactate; Invitrogen). For the assays using mCherry-expressing *C. burnetii*, the cells were washed twice with room temperature PBS after the fixation and then directly mounted with Prolong Gold antifade reagent containing DAPI.

Multiphoton microscopy images were acquired using an LSM 780 Zeiss AxioObserver microscope equipped with a 63× oil immersion objective and analyzed using Imagel software.

**Transmission electron microscopy.** AMs were infected for 3 or 6 days, washed once with PBS, fixed for 1 h at room temperature with modified Karnovsky’s fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4), dislodged by scraping, and then processed for and analyzed by transmission electron microscopy as previously described (7).

**Quantification of *C. burnetii*.** BMDMs, AMs, THP-1 cells, and irradiated Vero cells infected with *C. burnetii* were maintained in culture for up to 12 days. After 6 days of infection, cells were supplemented with an additional 500 µl of RPMI 1640 medium with 10% FBS and 5% LCCM for...
BMDMs, RPMI 1640 medium with 10% FBS for AMs and THP-1 cells, and DMEM (Gibco) with 10% FBS (Gibco) and 1 mM t-glutamine (Sigma-Aldrich) for irradiated Vero cells. At different times postinfection, cells were lysed with 1.0 ml of deionized water and cell lysates were combined with the culture supernatant from the respective wells. The material was centrifuged at 14,000 × g for 15 min at 4°C for deposition of the bacteria present in the samples. The DNA from C. burnetii was extracted using a Bacteria GenomicPrep Illustri kit (GE Healthcare), and the presence of genetic material was confirmed by spectrophotometry (determination of the absorbance at 260 nm). The primer pair dotA-for and dotA-rev (18) was used for quantitative PCR (qPCR), a reliable method for quantification of bacterial replication, as demonstrated by Brennan and Samuel (16) for analyses involving C. burnetii. The reaction was performed using a Platinum SYBR green qPCR Supermix-UDG with carboxy-X-rhodamine (ROX) kit (Invitrogen), and thermocycling reactions were performed according to the manufacturer’s instructions. The samples were read on an ABI Prism 7000 thermocycler.

RT-PCR analysis. mRNA from cultures of 10^7 AMs and BMDMs derived from C57BL/6 mice noninfected or infected for 12 h with C. burnetii at an MOI of 3 were extracted with the TRIZol reagent. RNA purification was done using an Illustra RNAspin minikit (GE Healthcare), according to the manufacturer’s instructions, and genetic material was quantified using a NanoDrop spectrophotometer (Thermo Scientific). ImProm-II reverse transcriptase (Promega) was used to obtain cDNA. The real-time quantitative PCR assays were performed using the Platinum SYBR Green qPCR Supermix-UDG with carboxy-X-rhodamine kit (Invitrogen). Thermocycling reactions were performed according to the manufacturer’s instructions, and the samples were read on a StepOnePlus real-time PCR system (Applied Biosystems). The expression of the target genes was calculated using the mean threshold cycle (C_T) values of each sample, which were normalized to those for the housekeeping genes. The sequences of the primers used for reverse transcription-PCR (RT-PCR) are as follows: Gapdh-fwd, 5'-CATCACCATCTTCTCAGGACGG-3'; Gapdh-rev, 5'-ACGGACACATTGGGGGTAGG-3'; Nos2-fwd, 5'-CGAAGAACGC TTCSCTTCAA-3'; Nos2-rev, 5'-TGAGCCCTATTGCTGTGGCT-3'; Tnf-fwd, 5'-GCACCTGGCCTTGGACGCT-3'; Tnf-rev, 5'-CCTTTTTCG CCTTGGCTGTG-3'; Tnf-rev, 5'-TGTGTCAGACGCTTTCAACA-3'; Tnf-fwd, 5'-CTTGTATGCTGATGAGA-3'; Th2-fwd, 5'-AAGTCTCC GAAATATCATGTC-3'; Th2-rev, 5'-TGAAGATGATGCGGCAAT-3'; Il4-fwd, 5'-AAGAGCGATCATCAAGGAG-3'; Il4-rev, 5'-TAAAGGCT GGGTGCCCTGAC-3'; Arginase1-fwd, 5'-GTTCCAGATCAGCCATGACTC-3'; Arginase1-rev, 5'-CGATGTCTTTGGAGATATGC-3'; Fizz1-fwd, 5'-TGAGCCTTGCTGGTGGAG-3'; Fizz1-rev, 5'-CTTCTG ACAGTTGTCAGACG-3'; Ym1-fwd, 5'-GGGGTGTATTGACAATGT CAG-3'; and Ym1-rev, 5'-TGAAGTCTCATCTGGTACAGG-3'.

Flow cytometry for phenotypic analysis. Single suspensions of AMs and BMDMs from C57BL/6 mice were stained with stains from the LIVE/DEAD fixable blue dead cell stain kit (Invitrogen) in PBS to exclude dead cells. Cells were stained with fluorochrome-conjugated antibodies against surface markers CD11b (clone M1/70), F4/80 (clone BM8), CD206 (clone C068C2), and CD86 (clone GL-1), purchased from BD Biosciences (San Diego, CA), and dectin-1/CLEC7A (CD69), obtained from BioLegend, in PBS containing 1% FBS for 30 min at 4°C and then washed. Staining was performed in the presence of 5 μg/ml of Fc Block (BD Biosciences). Cell acquisition was performed on a BD FACSCanto II flow cytometer using FACSDiva software (BD Biosciences). For each sample, at least 100,000 events were collected. Data were analyzed with FlowJo software (TreeStar).

Statistical analyses. Statistical analyses were performed using GraphPad Prism (version 5) software. The results were analyzed by a statistical test for analysis of variance (ANOVA) followed by the Bonferroni posttest or Student’s t test. Differences were considered statistically significant when the P value was <0.05.

RESULTS
Alveolar macrophages from C57BL/6 mice are permissive to replication of C. burnetii. We have previously shown that BMDMs from A/J and BALB/c are less restrictive to the replication of C. burnetii phase II than cells from mice with the C57BL/6 mouse genetic background. This was determined by assessing the formation of the large CCV and by the determination of the number of C. burnetii focus-forming units (9). To further confirm these data, we quantified the bacterial genomic DNA by quantitative PCR (qPCR) as a tool to assess the replication of C. burnetii in BMDMs as previously described (16). In agreement with previously published data (9), we found that BMDMs from C57BL/6 mice are highly restrictive to C. burnetii phase II, whereas BMDMs from A/J mice support some intracellular replication of C. burnetii phase II from days 3 to 12 postinfection. This was observed in experiments using high and low infection doses (MOIs, 100 and 3, respectively) (Fig. 1A and B, respectively). We continued the macrophage infection studies using an MOI of 3. Because most of the gene-deficient mice generated thus far were constructed in the C57BL/6 mouse background, we aimed to identify a primary cell that supported the replication of C. burnetii phase II in culture. It is known that C. burnetii infects macrophages and monocytes, with AMs considered the primary target cell of infection (10, 12, 19–22). Thus, we evaluated whether murine AMs from the C57BL/6 mouse strain support the replication of C. burnetii phase II in culture. Strikingly, when we assessed bacterial replication using qPCR, we found that, as opposed to BMDMs, the AMs were highly permissive to C. burnetii phase II replication (Fig. 1C). Of note, the internalization of bacteria by these different cells was similar, as measured by determination of the number of C. burnetii genomes associated with the cells after 4 h of infection (Fig. 1A to D, insets). As a negative control for bacterial replication, rifampin treatment was included to kill the C. burnetii bacteria in the macrophages. Further, when we compared the C. burnetii replication in AMs from C57BL/6 and A/J mice, we found that AMs from both strains were equally permissive for C. burnetii replication (Fig. 1D). These data suggest that the genetic mechanisms controlling the natural resistance of BMDMs to C. burnetii may be less effective or absent in AMs. Importantly, AMs emerge as a very interesting model of the primary macrophage that supports the intracellular replication of C. burnetii phase II in culture. As such, we decided to further characterize C. burnetii replication in AMs.

Alveolar macrophages accumulate large amounts of intracellular bacteria within the Coxiiella-containing vacuole. Given the robust replication of C. burnetii phase II in murine AMs (Fig. 1C and D), we aimed to assess bacterial replication in these macrophages by microscopy. AMs obtained from C57BL/6 mice were infected, and intracellular bacteria were visualized by immunofluorescence and transmission electron microscopy. Figure 2 illustrates the susceptibility of AMs over the first 6 days of C. burnetii phase II infection in culture. We detected an increased concentration of intracellular C. burnetii, stained in red, during the course of infection, suggesting that the bacteria can readily replicate in AMs. Further, the association of the bacteria with the lysosomal membrane marker LAMP-1 confirms that the bacteria replicate in the well-characterized lysosome-derived parasitophorous vacuole. During the procedures to stain the bacteria and LAMP-1, the AMs containing large spacious vacuoles were lost because these cells easily detach from the coverslips during the antibody washings.
Thus, to assess the presence of typical large vacuoles in infected AMs, we used a strain of *C. burnetii* expressing mCherry (23). By examining the infected AMs using two-photon laser scanning fluorescence microscopy, we found a significant increase in bacterial replication in AMs from days 0 (4 h of infection) to 12 after infection (Fig. 2A and B). At day 6 of infection, we observed several cells containing many *C. burnetii* bacteria in large vacuoles (Fig. 2B). At day 9 of infection, the AMs were filled with *C. burnetii* bacteria, as illustrated by a merge image showing both differential interference contrast and fluorescence images (Fig. 2C). Intracellular bacteria were also observed by transmission electron microscopy (Fig. 3). At 3 days after infection, AMs showed large CCVs containing high numbers of large-cell variants of *C. burnetii* (Fig. 3A). At day 6 after infection, we detected vacuoles harboring a large amount of *C. burnetii* bacteria (Fig. 3B). Taken together, the results confirm that AMs support *C. burnetii* replication in culture and can be used as a useful model of the primary macrophage to study *C. burnetii* phase II replication in vitro.

Alveolar macrophages show a more pronounced polarization into M2 cells than bone marrow-derived macrophages. To
assess the mechanisms underlying the permissiveness of AMs to C. burnetii, we evaluated M1/M2 polarization, which has previously been shown to be important for C. burnetii replication and pathogenesis (24, 25). Initially, we evaluated the expression of genes known to be hallmarks of M1 and M2 polarization in AMs and BMDMs. By measuring gene expression in noninfected BMDMs and AMs, we found that the latter show a significantly reduced expression of \textit{Nos2}, \textit{Tnfa}, \textit{Ifng}, and \textit{Tlr2} (genes associated with an M1 polarization phenotype) and an increased expression of \textit{Arginase1}, \textit{Fizz1}, and \textit{Ym1}, which are associated with an M2 profile (Fig. 4). The AMs also showed a pronounced M2 polarization compared to that of BMDMs in response to C. burnetii infection. We detected reduced expression of \textit{Nos2}, \textit{Tnfa}, \textit{Ifng}, and \textit{Tlr2} and increased expression of \textit{Il4}, \textit{Arginase1}, \textit{Fizz1}, and \textit{Ym1} in AMs infected with C. burnetii compared to their expression in infected BMDMs (Fig. 4). These data are consistent with AMs being more susceptible because they polarize toward an M2 phenotype. Accordingly, M1 macrophages effectively trigger the production of tumor necrosis factor alpha (TNF-\alpha), a cytokine known to facilitate the restriction of C. burnetii replication in macrophages (26). To further test this hypothesis, we evaluated the expression of surface markers known to be hallmarks of M2 macrophages, including mannose receptor (CD206) and dectin-1 (27). To evaluate protein expression, we performed fluorescence-activated cell sorting experiments in which we gated live macrophages and evaluated the expression of the cell surface markers CD206, dectin-1, and CD86 on cells double positive for CD11b and F4/80 (Fig. 5A and B). Using specific antibodies, we found that AMs showed increased expression of M2-associated molecules, such as CD206 (mannose receptor) and dectin-1 (CLEC7A), compared to BMDMs. This was evident under the uninfected condition (Fig. 5C) and in response to infection (Fig. 5D). Moreover, we measured the expression of the costimulatory molecule CD86, which was similarly expressed in AMs and BMDMs (Fig. 5C and D). These data are consistent with the hypothesis that AMs are more susceptible because of an M2 polarized phenotype, a characteristic that has previously been associated with permissiveness to C. burnetii replication (reviewed in reference 28).

\textbf{Alveolar macrophages deficient in NOS2 and IFN-\gamma are more susceptible to C. burnetii replication, whereas cells deficient in interleukin-4 (IL-4) are more restrictive to C. burnetii replication.} It is well established that nitric oxide (NO) is involved in the restriction of C. burnetii replication (8, 9, 29, 30). Thus, we assessed bacterial replication in AMs deficient in nitric oxide synthase 2 (NOS2; or inducible nitric oxide synthase [iNOS]) as a proof of principle that AMs can be used as a model to investigate the interactions of C. burnetii with primary mouse macrophages in the C57BL/6 mouse genetic background. In agreement with previous reports, we found that AMs from Nos2\textsuperscript{-/-} mice are more permissive to C. burnetii phase II replication than AMs from wild-type C57BL/6 mice (Fig. 6A). We also evaluated bacterial replication in AMs from Ifng\textsuperscript{-/-} mice because gamma interferon (IFN-\gamma) is known to be essential for NO production (31). Moreover, IFN-\gamma is well described to be involved in the immune re-
FIG 4 Alveolar macrophages exhibit higher levels of expression of genes related to the M2 phenotype. BMDMs and AMs from C57BL/6 mice were left uninfected or infected for 12 h with C. burnetii at an MOI of 3. Total RNA was extracted, and reverse transcription-PCR (RT-PCR) was performed for detection of the genes Nos2, Tfna, Il4, Tlr2, Ifng, Arginase1, Fizz1, and Ym1. The data shown are the means ± standard errors from one representative experiment out of two performed. *, P < 0.05 comparing BMDMs and AMs; #, P < 0.05 comparing infected cells with the uninfected cells from the respective group (two-way ANOVA).

response against C. burnetii (19, 32–40). We found that AMs from Ifng−/− mice are significantly more permissive to C. burnetii phase II replication than AMs from wild-type C57BL/6 mice (Fig. 6B). Collectively, these data support the use of AMs as a model of primary macrophages for investigations involving C. burnetii infections in primary macrophages from the C57BL/6 mouse, the strain mostly commonly used for the generation of transgenic mice. To further understand the effect of the M2 polarization of AMs that results in the increased susceptibility to C. burnetii, we performed experiments using AMs from mice deficient in IL-4, which is required for M2 differentiation (reviewed in reference 41). We found that AMs from Il4−/− mice are less permissive to C. burnetii replication than cells from C57BL/6 mice (Fig. 6C). The internalization of bacteria did not differ in AMs from C57BL/6, Nos2−/−, Ifng−/−, and Il4−/− mice, as measured by the number of C. burnetii bacteria in cultures infected for 4 h (Fig. 6A to C, insets). Collectively, these data support the hypothesis that the M1/M2 polarization underlies the increased susceptibility of AMs in comparison to BMDMs. Importantly, regardless of the mechanisms associated with the resistance of the BMDMs, these data support the use of AMs as an important and relevant host cell for evaluation of C. burnetii replication in primary macrophages.

The permissiveness of mouse alveolar macrophages is comparable to that of highly susceptible immortalized cell lines. Our results demonstrated that murine AMs are permissive for C. burnetii phase II replication in vitro. Thus, we decided to compare C. burnetii phase II replication in C57BL/6 mouse AMs with that in highly susceptible host cells, such as Vero cells (a green monkey kidney cell line) and cells of a monocytic cell line (THP-1) differentiated into macrophage-like cells. Both of these cell types are known to be very susceptible to C. burnetii replication (6, 14, 34). Figure 7 shows that at 6 days postinfection, murine AMs are as susceptible to C. burnetii phase II infection as Vero and THP-1 cells. At later time points after infection, the Vero and THP-1 cell lines supported bacterial replication more robust than that in primary AMs. Nonetheless, the comparable rate of replication during the first 6 days of infection reinforces the demonstration that murine AMs are indeed highly permissive for C. burnetii replication and can be used as a relevant host cell model for investigations involving the macrophage interaction with C. burnetii phase II in culture.

DISCUSSION

C. burnetii is a unique intracellular bacterial pathogen capable of replicating to high numbers within a lysosome-derived vacuole. Humans can contract Q fever through the inhalation of contaminated aerosols and the subsequent intracellular replication of C. burnetii within AMs. As such, studying the interaction between
this specific macrophage lineage and the pathogen provides an important, disease-relevant, in vitro model to understand the specific host-pathogen interaction that takes place during *C. burnetii* infection. Previous research has demonstrated that BMDMs from different mouse strains vary in their permissiveness to *C. burnetii* replication (42). BMDMs from A/J and BALB/c mice support the intracellular replication of *C. burnetii*, while BMDMs from C57BL/6 mice restrict bacterial replication. Here, we recapitulate these results and demonstrate that AMs are significantly more permissive to *C. burnetii* replication than BMDMs. This finding is most striking with C57BL/6 mice, in which BMDMs restrict the intracellular growth of *C. burnetii*, yet the bacteria readily replicate in AMs. Indeed, despite a remarkable difference in the replication of *C. burnetii* in BMDMs of C57BL/6 and A/J mice, there is no

**FIG 5** Alveolar macrophages exhibit an M2 phenotype. (A and B) Gating strategy and frequency of noninfected BMDMs (A) and AMs (B). FSC, forward scatter; SSC, side scatter. (C and D) Surface phenotyping of BMDMs and AMs infected (for 24 h) (C) or not infected (D) with *C. burnetii* for CD86 and the M2 markers CD206 and dectin-1, as analyzed by flow cytometry. Cells were gated on live CD11b+ F4/80+ cells.
significant difference in the ability of *C. burnetii* to replicate in AMs from these two inbred mouse strains. This suggests that the genetic basis for C57BL/6 mouse BMDM restriction of *C. burnetii* infection reflects phenotypic features that may be specific for this cell type or that operate at a lower efficiency in AMs.

AMs are critical lung resident cells that, in healthy lungs, constitute the vast majority of the cellular content within alveoli (43). As such these cells are intrinsically essential for lung homeostasis and immune surveillance (44). Recent evidence indicates that AMs originate from fetal monocytes that are long living and are maintained by self-renewal rather than circulating bone marrow-derived monocytes (45). This tissue microenvironment has a significant epigenetic and transcriptomic impact on resident macrophages, rendering AMs phenotypically distinct from BMDMs (46–48). In light of our results, one can suggest that these phenotypic differences between BMDMs and AMs from C57BL/6 mice mediate a significant difference in the capacity to restrict the intracellular replication of *C. burnetii*. Interestingly, our data comparing M1/M2 polarization in BMDMs and AMs suggest that the M2 profile of AMs may underlie their increased susceptibility to infection. This hypothesis is supported by experiments using cells from *Ifng<sup>−/−</sup>* mice. In the absence of IL-4, the AMs became significantly more resistant to infection, a feature that is consistent with the essential role of IL-4 in the induction of M2 polarization (41).

In support of our data showing the increased replication of *C. burnetii* in AMs, it was previously reported that tissue-resident AMs are susceptible to *C. burnetii* replication *in vivo* (12). Moreover, human AMs were shown to support *C. burnetii* replication in culture (11). In addition, a system that allows infection of ex *vivo* human lung tissue was recently reported. It was found that within human lung tissue, *C. burnetii* preferentially replicated in human AMs (49). In this context, our data provide a definitive demonstration that mouse AMs can be used as a relevant host cell for investigations of the interactions of *C. burnetii* with host cells *in vitro*; C57BL/6 mouse AMs support the intracellular replication of *C. burnetii*, and the bacteria replicate in a manner that is comparable to that in widely used immortalized cell lines that are highly susceptible to *C. burnetii* replication. After the first 6 days after infection, we found that *C. burnetii* phase II replicates similarly in AMs, THP-1 cells, and Vero cells. At days 9 and 12, we detected higher bacterial loads in Vero and THP-1 cells, which are possibly caused by the saturation of the host cells available for bacterial replication. This hypothesis is supported by the experiments performed with AMs infected with mCherry-expressing *C. burnetii*. After 9 days of infection, we visualized AMs filled with bacteria, which were contained in large vacuoles that occupy the entire

**FIG 7** Alveolar macrophages are permissive to *C. burnetii* phase II replication, similar to Vero and THP-1 cells, *in vitro*. C57BL/6 AMs, irradiated Vero cells, and THP-1 cells (previously differentiated overnight with 200 nM PMA) were infected with *C. burnetii* phase II (MOI, 3), and bacterial multiplication was evaluated by quantification of the genomic DNA by qPCR after 0 (4 h after infection), 3, 6, 9, and 12 days of infection. The y axis represents the difference in the base 10 logarithm between the genomic quantity X at time t [logX(t)] and the quantity X at time zero (T<sub>0</sub>) [logX(0)]. Cells treated with rif were used as a negative control of bacterial replication. (Insets) Amount of *C. burnetii* genomic DNA present in cells after 4 h of infection. The data shown are the means ± standard deviations from one representative experiment out of three independent experiments performed. *, P < 0.05 (two-way ANOVA).
macrophage cytoplasm (Fig. 2B and C). We did not detect a reduction of bacterial loads from days 9 to 12, a feature that does not support the restriction of bacterial replication in the AMs at later stages of infection.

C57BL/6 mouse AMs can serve as an important host cell for the investigation of host factors that aid or restrict the intracellular replication of C. burnetii. Here we have confirmed the importance of both nitric oxide and IFN-γ in restricting the ability of C. burnetii to replicate. Significantly higher levels of C. burnetii were recovered from both Nos2−/− and Ifng−/− mouse AMs than from wild-type C57BL/6 mouse AMs. Production of nitric oxide and its reactive nitrogen intermediates is an important cellular response that acts against respiratory pathogens (50). At high levels, nitric oxide has a cytotoxic effect and can participate in the control of intracellular pathogen replication. Indeed, several studies have demonstrated that nitric oxide has the capacity to restrict the intracellular replication of C. burnetii (8, 9, 29, 30, 51). Our data support these findings, demonstrating that AMs from C57BL/6 mice lacking Nos2 support higher levels of C. burnetii replication than wild-type cells. Similarly, the absence of IFN-γ, which is critical for nitric oxide production, is known to be important to host restriction of C. burnetii replication. Importantly, by using AMs from Nos2−/− and Ifng−/− mice, we validated the use of AMs as a relevant host cell for the in vitro infection setting. Our characterization of this model of infection demonstrates that studying the interaction between C. burnetii and AMs will allow intricate investigation of both host and bacterial factors that are important for pathogenesis and host resistance during Q fever.

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