Virulent *Coxiella burnetii* does not activate human dendritic cells: Role of lipopolysaccharide as a shielding molecule

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**Coxiella burnetii** is an obligate intracellular bacterium and the etiological agent of the zoonotic disease Q fever. Acute human Q fever is characterized by flu-like symptoms that, in some cases, can result in a persistent infection that may reactivate months or years after initial exposure. Mechanisms by which this obligate parasite evades clearance by the host immune response during persistent infection are unknown. Here, we characterized the interaction of *C. burnetii* with dendritic cells (DC), critical components of both innate and adaptive immunity. Human DC were infected with two isogenic *C. burnetii* strains that differ in LPS length. Infection by the Nine Mile phase I (NMI) strain, which is fully virulent and produces full-length LPS, did not result in DC maturation. In contrast, infection by the avirulent Nine Mile phase II strain, producing a severely truncated LPS, resulted in toll-like receptor 4-independent DC maturation and ~10-fold more IL-12 and TNF production. NMI did not actively inhibit DC maturation as NMI-infected DC subsequently matured if treated with *Escherichia coli* LPS or Nine Mile phase II. Furthermore, removal of LPS from NMI dramatically reduced immune evasion | Q fever | toll-like receptor | persistence | phase variation

**T**he obligate intracellular, Gram-negative bacterial pathogen *Coxiella burnetii* infects a wide range of vertebrate hosts. Acute infection of humans, termed Q fever, results in nonspecific, flu-like symptoms such as high-grade fever and severe preorbital headache. *C. burnetii* can also establish a persistent infection that may later reactivate and result in chronic endocarditis or hepatitis (1). Human infection usually occurs via the aerosol route and is most commonly associated with exposure to infected livestock or their products (1). Additionally, *C. burnetii* has an extremely low infectious dose (<10 organisms) (2) and is very stable in the environment, which has led to its designation as a potential bioterrorism agent by the U.S. Centers for Disease Control and Prevention.

The obligate intracellular nature of *C. burnetii*’s lifestyle has precluded the establishment of a system to genetically study the virulence of this organism. *C. burnetii* strains of decreased or no virulence in animal models have been generated through serial in vitro passage. These isogenic strains have undergone a “phase variation” event in which a chromosomal mutation affecting LPS biosynthesis has occurred (3). Avirulent or “phase II” bacteria, typified by the Nine Mile phase II (NMII) strain, produce an LPS that lacks the unique branched terminal sugar-containing O-polysaccharide chain that is characteristic of the LPS of virulent or “phase I” organisms, such as the Nine Mile phase I (NMI) strain (4). Phenotypically, NMI differs from NMII *C. burnetii* in that only NMI causes disease and persists in a guinea pig model of infection (2). Moreover, NMI is more resistant than NMII to complement-mediated serum killing (5). Finally, uptake of NMI by monocytes appears to be mediated by the leukocyte response integrin (*α*2*β*1), whereas uptake of NMII is mediated by both *α*2*β*1 and complement receptor 3. This behavior has been implicated in differential intracellular trafficking of NMI and NMII (6).

The acute form of Q fever appears to stimulate an efficient immune response that eventually limits bacterial replication but fails, in many cases, to completely clear the pathogen. Indeed, a recent study by Marmion et al. (7) detected *C. burnetii* DNA in the bone marrow of 88% of patients 12 years after acute primary Q fever. Persistent, asymptomatic infection of animals by *C. burnetii* is also well documented (8). Viable *C. burnetii* has been recovered from animal tissues up to 500 days postinfection, and latent infection in guinea pigs can be reactivated by pregnancy, irradiation, or corticosteroid treatment (9–11).

Peripheral blood lymphocytes (PBLs) from convalescent acute disease patients proliferate in response to *C. burnetii* antigens (12). However, PBLs from patients with chronic disease fail to proliferate after exposure to the same antigens, and a suppressor/regulatory T cell population appears to be involved in this phenomenon (12, 13). Additionally, cytokine-mediated down-regulation of macrophage function may play a role in *C. burnetii* persistence (14, 15). Collectively, these observations indicate the presence of an unknown mechanism of suppression of cell-mediated immunity to *C. burnetii*.

Activated dendritic cells (DC) express high levels of MHC class II and T cell costimulatory molecules, giving them the unique ability to efficiently stimulate naïve T cells (16, 17). DC reside in the peripheral epithelial tissues in an immature state (iDC) where they serve as sentinels against invading microorganisms (18). Contact with a pathogen typically results in stimulation of pattern recognition receptors such as the toll-like receptor (TLR) and subsequent conversion of iDC into mature DC. DC maturation is marked by a reduction in phagocytic ability, an increase in surface expression of MHC class II and costimulatory molecules, and changes in chemokine receptor expression resulting in DC migration to the local lymph nodes (16, 19, 20).

The attributes of DC place them at the interface of innate and adaptive immunity. Consequently, pathogens have developed a variety of mechanisms to modify DC function as a means of influencing the host immune response in their favor (21–25). To determine whether infection by *C. burnetii* alters DC function, we characterized the interaction of two strains of *C. burnetii*,

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Abbreviations: DC, dendritic cells; MAPK, mitogen-activated protein kinase; MOL, multiplicity of infection; NMI, Nine Mile phase I; NMII, Nine Mile phase II; TCA, trichloroacetic acid; Th1, T helper 1; TLR, toll-like receptor.

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producing full-length or truncated LPS, with human monocyte-derived DC.

Materials and Methods

C. burnetii and Reagents. C. burnetii NMI (RSA493) and NMII (RSA439) strains were propagated in Vero cells. Organisms were purified from infected cells by Renografin density gradient centrifugation (26). Purified bacteria were suspended in K-36 buffer (0.1 M KCl/0.015 M NaCl/0.05 M K$_2$HPO$_4$/0.05 M KH$_2$PO$_4$, pH 7.0). Trichloroacetic acid (TCA) extraction of LPS was performed as described (27). Briefly, purified NMI or NMII bacteria were suspended in TCA solution (10% TCA in 0.14 M NaCl) at a concentration of 1 mg/ml for 4 h at 4°C with occasional mixing. Cells were pelleted by centrifugation at 12,000 g for 10 min, and the extraction was repeated. LPS from Escherichia coli serotype O111:B4 was purchased from Sigma and used at 500 ng/ml. Anti-CD14 (clone 134620) antibody (R & D Systems) was used to characterize DC phenotypes: antiCD83-APC, antiCD86, antiCD63 and convalescent guinea pig C. burnetii. Human DC Undergo Maturation in Response to Avirulent, but Not Virulent, C. burnetii. To determine whether infection by virulent, C. burnetii induces DC maturation, immature human monocyte-derived DC were harvested by centrifugation at 500 × g for 5 min and resuspended in 100 µl of cell lysis buffer [1% PBS, 1% Triton X-100, and Complete Protease Inhibitor (Roche Molecular Biochemicals)]. Lysates were centrifuged at 10,000 × g for 10 min and diluted in 5× SDS/PAGE sample buffer. Samples were boiled for 5 min, and proteins were separated by SDS/PAGE on a 12% gel. Proteins were transferred to an Immobilon (Millipore) membrane, blocked overnight with 5% BSA in PBS, then probed with rabbit polyclonal anti-phosphoP38 (Cell Signaling Technology, Beverly, MA) and anti-rabbit IgG-horseradish peroxidase (HRP) antibodies. HRP was detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The membrane was then incubated for 15 min at 37°C in Restore Western Blot Stripping Buffer (Pierce) and reprobed with rabbit polyclonal anti-totalp38 (Cell Signaling Technology) and anti-rabbit IgG-HRP antibodies to normalize for sample loading.

Quantitative PCR. C. burnetii replication during infection of DC was quantified by using TaqMan quantitative PCR of genome equivalents as described (28). Briefly, DC in suspension were incubated with NMI or NMII C. burnetii for 2 h at room temperature with gentle rocking. Nonadherent bacteria were removed by washing infected DC once with Hanks’ balanced salt solution, followed by centrifugation at 200 × g for 5 min. Infected DC were replated in 6-well plates at 2 × 10⁵ cells per well. This point was considered 0 h postinfection. DC were harvested by scraping, and bacterial DNA was isolated with an UltraClean microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The primers/probe set used was designed with PRIMER EXPRESS software (Applied Biosystems) and is specific for the C. burnetii dotA gene (28). The forward and reverse primers are GCGATACCGGCGGTGGG and CCG-GCGCAATACGCTCAATCACA and the probe sequence is CCG-GCGCAATACGCTCAATCACA and CCATGGCCCCAACAT-TCTCTT, respectively, and the probe sequence is CCG-GAGATACCGGCGGTGGG. Purified plasmid DNA containing the C. burnetii dotA gene was used as template to generate standard curves ranging from 10³ to 10⁸ plasmid copies.

Cytokine Measurement. IL-12p70 and TNF concentrations in culture supernatants were determined by using OptEIA ELISA kits (BD Pharmingen).

Results

Human DC Undergo Maturation in Response to Avirulent, but Not Virulent, C. burnetii. To determine whether infection by C. burnetii NMI or NMII strains induces DC maturation, immature human monocyte-derived DC were infected for 48 h, then analyzed by flow cytometry for surface expression of the maturation markers CD80, CD83, CD86, CD40, and HLA-DR. Interestingly, only infection by avirulent NMII induced DC maturation (Fig. 1). Because NMII C. burnetii is internalized ∼10-fold more effi-
ciently than NMI by a wide variety of cell types (2), we investigated whether uptake efficiencies could explain the differential maturation of DC in response to *C. burnetii* infection. The DC response (i.e., the lack of maturation) to NMI infection at an MOI of 200 was indistinguishable from infections at an MOI of 20. These data suggest that LPS length is a major determinant of the effects of *C. burnetii* strains on DC.

**Human DC Produce Inflammatory Cytokines in Response to Avirulent, but Not Virulent, *C. burnetii***. IL-12 production by DC is essential in driving a T helper 1 (Th1) immune response, which is important for clearance of intracellular pathogens. TNF is a multipotent inflammatory cytokine important for defense against a variety of intracellular pathogens (29). We therefore determined whether *C. burnetii* strains could induce IL-12 and TNF production by DC in vitro. Similar to its ability to induce DC maturation, NMII also consistently stimulated at least 10-fold more IL-12 and TNF production than virulent NMI *C. burnetii* (Fig. 2). Several pathogens modulate host immune responses by inducing IL-10 production by DC, and IL-10 levels are increased in some chronic Q fever patients (15). However, we observed no significant IL-10 production by NMI- or NMII-infected DC when compared with mock-infected controls (data not shown).

**Infection of DC with NMII *C. burnetii* Results in Increased p38 Mitogen-Activated Protein Kinase (MAPK) Phosphorylation**. Many bacterial pathogens induce DC maturation via TLR ligation. This ligation results in a cascade of signaling events, including the phosphorylation of cellular p38 MAPK (30). To determine whether NMII-induced DC maturation might be caused by TLR stimulation, we examined extracts of infected DC by immunoblot to determine the amount of phosphorylated p38 MAPK present. Infection with NMII resulted in a marked increase in phosphorylated p38 MAPK present. In contrast, infection with the virulent NMI strain had no effect on p38 phosphorylation as compared with mock-infected DC. We can conclude that infection with avirulent NMII *C. burnetii* results in TLR-associated cell signaling events that do not occur during infection with NMI.

**C. burnetii Productively Infects Human DC**. We next determined whether both virulent and avirulent *C. burnetii* could productively infect human DC. DC were infected with either NMI or NMII for 48 h, then immunostained for *C. burnetii* and the lysosomal marker CD63. Fig. 4A shows that both strains formed large, spacious, CD63-positive vacuoles that appear indistinguishable from vacuoles formed in other cell types known to be permissive for *C. burnetii* growth (31, 32).

Formation of large, spacious vacuoles and the beginning of
exponential growth phase of *C. burnetii* coincide in other cell types (28). To examine the growth characteristics of NMI and NMII *C. burnetii* in DC, we used quantitative PCR to measure the increase in *C. burnetii* genomes over time. Both NMI and NMII replicated in DC with nearly identical growth kinetics (Fig. 4B). A typical 48-h lag phase was observed, followed by exponential growth that resulted in ~26- and 47-fold increases in NMI and NMII genomes, respectively. These results are comparable to growth rates observed in other cell types (28, 33).

**NMI *C. burnetii* Does Not Actively Inhibit DC Maturation.** To determine whether the maturation of DC was being actively inhibited by NMI *C. burnetii*, DC were infected for 18 h with NMI, then stimulated with *E. coli* LPS, infected with NMII, or mock-infected. The DC were then incubated for an additional 24 h, and their maturation status was determined by measurement of CD86 expression by flow cytometry (Fig. 5). Infection by NMI did not inhibit DC maturation in response to subsequent LPS treatment or infection with NMII. A similar maturation response was observed when DC were infected simultaneously with NMI and NMII (data not shown). These data indicate that the immature phenotype of DC infected with NMI *C. burnetii* is caused by a lack of stimulation rather than an active inhibition of maturation by the bacteria.

**DC Maturation in Response to NMII *C. burnetii* Is CD14-Independent.** Because NMI and NMII *C. burnetii* are known to differ only in LPS length, and LPS from a variety of bacterial pathogens signal through TLR4, we wanted to determine whether TLR4 signaling was involved in the response of DC to NMII. Antibodies are used to block signaling by several TLRs (34). However, human DC are

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**Fig. 2.** DC produce IL-12 and TNF in response to infection by NMII, but not NMI *C. burnetii*. Human DC were either mock-infected or infected (MOI = 20) for 48 h with *C. burnetii* NMI or NMII. Cell culture supernatants were collected, and their IL-12p70 and TNF concentrations were determined by ELISA. The results shown are from one experiment and are representative of three independent experiments.

**Fig. 3.** p38 MAPK is phosphorylated in DC in response to infection by NMII *C. burnetii*. Human DC were either mock-infected or infected (MOI = 20) with *C. burnetii* NMI or NMII for 48 h. (Upper) Proteins in cell extracts were separated by SDS/PAGE, blotted, and probed with anti-phospho-p38 MAPK antibody. (Lower) Blots were then stripped and reprobed for total p38 MAPK with anti-p38 MAPK antibody. Results shown are representative of three independent experiments.

**Fig. 4.** *C. burnetii* NMI and NMII productively infect human DC. (A) Human DC infected for 48 h were stained by indirect immunofluorescence for *C. burnetii* (green) and CD63 (red). Micrographs depict spacious CD63-positive vacuoles harboring NMI or NMII bacteria. The fields shown here are representative of the majority of the sample. The percentage of infected, vacuole-containing cells approached 100%. (Bars: 5 μm.) (B) Human DC were infected with either NMI (MOI = 100) or NMII (MOI = 10) as described in Materials and Methods. The number of bacterial genomes at 0, 48, 96, 144, and 192 h postinfection was determined by quantitative PCR. The results shown are from one experiment performed in triplicate and are representative of three independent experiments.

**Fig. 5.** NMI *C. burnetii* does not actively inhibit DC maturation. DC were either mock-infected or infected with NMI (MOI = 20) for 18 h, then both samples were left untreated (black histograms), treated with LPS (blue histograms), or infected with NMII (red histograms) (MOI = 20) for an additional 24 h. CD86 expression was measured by flow cytometry. (Left) Staining of cells that were mock-infected before LPS treatment or NMII infection. (Right) Staining of cells that were preinfected with NMI.
somewhat unique in that they express TLR4 only on internal membranes, not on the cell surface, which renders them inaccessible to blocking antibody (35). We therefore took advantage of the fact that soluble CD14 is required by DC for optimal LPS signaling through TLR4, and that this TLR4–CD14 interaction can be blocked in vitro with a specific antibody (36). Anti-CD14 antibody had no effect on NMII-induced maturation of DC as measured by increased expression of CD86 (Fig. 6 Left). In contrast, anti-CD14 antibody partially inhibited DC maturation in response to E. coli LPS (Fig. 6 Right). Thus, maturation of DC in response to NMII C. burnetii appears to be CD14-independent and by default TLR4-independent.

**TCA-Extracted NMI C. burnetii Induces DC Maturation.** To determine whether C. burnetii LPS is responsible for the differential maturation and activation of DC in response to NMI and NMII, we used a TCA extraction technique to remove LPS from the outer membrane of NMI C. burnetii. TCA is known to extract LPS from enterobacteria, and its ability to extract full-length C. burnetii LPS has been verified (27). TCA-extracted or unextracted NMI or NMII C. burnetii was added to the DC culture medium, and DC maturation was measured by flow cytometry after 24 h. Interestingly, DC underwent full maturation in response to TCA-extracted LPS (Fig. 7 Left), indicating that depletion of LPS had converted these organisms to the NMI phenotype. TCA-treated NMI retained its DC stimulatory capacity (Fig. 7 Right). Because the TCA extraction process kills C. burnetii, we tested the effects of formaldehyde-fixed NMI on DC. These bacteria did not induce maturation, indicating NMI does not need to be viable to avoid stimulating DC (data not shown). These results suggest full-length LPS of virulent C. burnetii prevents maturation of DC.

**Discussion**

We show here that NMI C. burnetii infects and grows within DC without subsequent maturation or activation of these cells. Remarkably, this organism can reside within DC in large numbers without being detected. Moreover, the lack of DC maturation is not a result of active inhibition of DC stimulation, as observed with other pathogens (21, 24, 37). DC maturation activates lysosomal proteases required for antigen processing and presentation (38) and increases the expression of costimulatory molecules necessary for the induction of T cell prolifer-

![Image](image_url)
does not require robust DC stimulation. However, Q fever is normally resolved in an otherwise healthy host (1). It totally evade the immune response by this mechanism, as acute induction of DC maturation. Interestingly, also masks TLR engagement of other bacterial surface components (21). The immune suppression that results from this blockade has been implicated as an important factor in allowing the colonization.

Shannonylation (data not shown). These data suggest LPS length is the regulatory capacity between NMI, S, and Priscilla strains in terms (41–43). However, we observed no difference in the DC stimulatory capacity of virulent C. burnetii remains to be defined.

The importance of DC in bridging the gap between innate and adaptive immunity is well documented (16). We present here a description of the effects of C. burnetii infection on DC. Taken together, our results show that the long, branched terminal LPS sugars of C. burnetii may allow the organism to avoid full activation of the adaptive immune response. This proposed “shielding” of bacterial TLR ligands by LPS represents a unique immune evasion strategy.

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