Stimulation of Toll-like Receptor 2 by Coxiella burnetii Is Required for Macrophage Production of Pro-inflammatory Cytokines and Resistance to Infection*

Received for publication, September 9, 2004, and in revised form, October 12, 2004
Published, JBC Papers in Press, October 13, 2004, DOI 10.1074/jbc.M410540200

Dario S. Zamboni,a,b Marco A. Campos,a Ana C. T. Torrecillas,d,e Kati Kiss,g James E. Samuel,f Douglas T. Golenbock,f Fanny N. Lauw,g,h Craig R. Roy, Igor C. Almeida,d,i,h and Ricardo T. Gazzinellia,b,c

From the aSection of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut 06536, bCentro de Pesquisas René Rachou, Oswaldo Cruz Foundation, Belo Horizonte MG 30190-002, the cDepartment of Parasitology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-000, Brazil, the dDepartment of Medical Microbiology and Immunology, Texas A & M University System, College Station, Texas 77843, the eDivision of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and the fDepartment of Biochemistry and Immunology, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte MG 31270-901, Brazil.

Innate and adaptive immune responses are initiated upon recognition of microbial molecules by Toll-like receptors (TLRs). We have investigated the importance of these receptors in the induction of pro-inflammatory cytokines and macrophage resistance to infection with Coxiella burnetii, an obligate intracellular bacterium and the etiological agent of Q fever. By using a Chinese hamster ovary/CD14 cell line expressing either functional TLR2 or TLR4, we determined that C. burnetii phase II activates TLR2 but not TLR4. Macrophages deficient for TLR2, but not TLR4, produced less tumor necrosis factor-α and interleukin-12 upon C. burnetii infection. Furthermore, it was found that TLR2 activation interfered with C. burnetii intracellular replication, as macrophages from TLR2-deficient mice were highly permissive for C. burnetii growth compared with macrophages from wild type mice or TLR4-deficient mice. Although LPS modifications distinguish virulent C. burnetii phase I bacteria from avirulent phase II organisms, electrospray ionization-mass spectrometry analysis showed that the lipid A moieties isolated from these two phase variants are identical. Purified lipid A derived from either phase I or phase II LPS failed to activate TLR2 and TLR4. Indeed, the lipid A molecules were able to interfere with TLR4 signaling in response to purified Escherichia coli LPS. These studies indicate that TLR2 is an important host determinant that mediates recognition of C. burnetii and a response that limits growth of this intracellular pathogen.

Coxiella burnetii is a Gram-negative, obligate intracellular bacterium that survives inside large replication vacuoles (LRVs) that display phagolysosomal characteristics such as low pH, the presence of lysosomal hydrolases and glycoproteins, and Rab7 on their membranes (1-4). Two phase variants of C. burnetii have been described, the highly virulent phase I and the avirulent phase II. Avirulent phase II C. burnetii can be obtained by multiple passages through chicken cells. Although avirulent for mammals, phase II organisms effectively infect and grow in cultured host cells (1, 2, 5). C. burnetii phase II, Nine Mile strain clone 4, contains a 26-kb deletion in the genome, which codes for a group of lipopolysaccharide biosynthetic genes (6), a feature that provides genetic support for the reported differences in the composition of LPS from C. burnetii phase I and II (7, 8).

C. burnetii phase I is the etiological agent of Q fever, a disease of worldwide distribution (9-11). Because of its ability to survive in the environment and high infectivity when aerosolized, C. burnetii is classified as a category B bioterrorism agent (12, 13). The disease manifests either as an acute or chronic illness with symptoms such as debilitating headache and cyclic fever (9). Acute Q fever is usually self-limiting in immunocompetent hosts, whereas the chronic form of the disease develops in individuals defective in cell-mediated immunity (9, 13). These findings support the fundamental role of an effective innate immune recognition to the host resistance against C. burnetii.

The innate immune system has evolved sophisticated mechanisms to sense invading microbes, to discriminate between different pathogens, and to initiate the production and secretion of inflammatory molecules that contribute to the development of an acquired immune response and host resistance to infection. Toll-like receptors (TLRs) constitute a family of pattern recognition molecules that can respond to molecular structures conserved in many microbial products. These transmembrane receptors contain ectodomains that have leucine-rich repeats that are involved in pattern recognition and intracellular signaling domains that initiate cellular responses to microorganisms.
phages from TLR4. To date, 11 functional TLRs have been described (TLR1–11). Many TLRs have the ability to respond to bacterial products (14–15). TLR2 appears to be the most promiscuous, responding to multiple bacterial products, including lipopolysaccharide (LPS), lipopeptides, lipoteichoic acid, and peptidoglycans (14, 15, 18). In contrast, TLR4 is specifically activated by the lipid A moiety of LPS from Gram-negative bacteria (19). How-

teringly, by a procedure that involves extensive delipidation followed by hot phenol (29) or, alternatively, by a polytrifluoroethylene method, previously used for extraction of glycolipids (27). LPS was preliminary purified with hot phenol (29) whereas Nine Mile strain phase II (RSA439) was additionally extracted (three times each) with 10 volumes of chloroform/methanol (2:1, v/v). Delipidated pellets were resuspended and counted as described (33). These cell lines expressing TLRs contain the CD25 gene under the control of E-selectin promoter, which contains an NF-κB-binding site. Thus, CD25 expression is dependent upon NF-κB activation (33, 34). Plates were plated (1 × 10^6 cells/well) in 24-well tissue culture dishes, cultured for 24 h, and stimulated with live C. burnetii phase II at a ratio of 10, 100, or 1000 bacteria per cell; 10, 100, 1000, or 1000 ng/ml of purified LPS; or 82 nmol (in regard to Lipid A). Controls included UV-killed E. coli (HB101) and Staphylococcus aureus (ATCC 12692), and LPS and lipid A extracted from E. coli and Bordetella pertussis. After 18 h stimulation, cells were stained with (R)-phycocerythrin-labeled anti-CD25 (mouse monoclonal antibody to human CD25, (R)-phycocerythrin conjugate; CALTAG Laboratories, Burlingame, CA) 1:200 and examined by flow cytometry (BD Biosciences) as described previously (35).

**TLR2 Signaling during Coxiella Infection**

Experimental Procedures

**Bacterial Preparation to Cellular Studies—Infective inocula of C. burnetii phase II.** Nine Mile strain clone 4 (RSA439) were prepared as described (27) from confluent Vero cells infected with C. burnetii for 7 days. Prior to infection, suspensions containing about 10^9 infective bacteria/ml were mildly sonicated at 35 kHz for 15 min at room temperature. Except for cytokine determination, macrophages were infected with about 100 infective organisms per cell. After 24 h, infected cultures were vigorously washed with Hank’s saline solution, and the appropriate fresh medium was added.

**LPS and Lipid A Extraction—C. burnetii.** Nine Mile strain phase I clone 7 (RSA493) was grown in Spf embryonated chicken eggs purified as described (28), whereas Nine Mile strain phase II (RSA439) was cultured and purified from persistently infected Vero cells as described (27). LPS was preliminarily purified with hot phenol (29) or, alternatively, by a procedure that involves extensive delipidation followed by butanol-water extraction, previously used for extraction of glycolipids and glycolipids from protozoan parasites (30). The latter procedure resulted in an LPS less contaminated with other bacterial components. Briefly, inactivated bacteria were lyophilized and sequentially extracted (three times each) with 10 volumes of chloroform/methanol (2:1, v/v, respectively) and chloroform/methanol/water (1:1, v/v). Lipid A was extracted with 10 volumes of 9% 1-butanol, for 4 h at room temperature, under constant shaking. Butanol extracts were grouped and dried using a rotary evaporator (Buchi, Switzerland), dissolved in endotoxin-free deionized water, and filtered through a 0.2-μm polytrifluoroethylene filter disk. Final purity of the LPS was determined by SDS-PAGE on a 12% gel, subsequently silver-stained as described previously (22). The lipid A moiety was isolated from LPS of phases I and II after mild acid hydrolysis with 0.25 N HCl (prepared from sequencing grade 6 N HCl) for 1 h at 100 °C, followed by neutralization with 0.25 N NaOH and Folch’s partition (31). Lipid A was recovered in the lower phase, dried under N2, and redisolved in chloroform/methanol (1:1, v/v), and quantified estimating the inorganic phosphate content (about 200 ng/ml).

**CHO Cell Lines and Flow Cytometry Analysis—**The CHO reporter cell lines (CHO/CD14, expressing functional TLR4; 7.19/CD14/TLR-2, expressing TLR2; and the 7.19 clone, expressing neither TLR2 nor functional MD2) were generated as described (33) and maintained as adherent monolayers in Ham’s F-12/Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, at 37 °C, 5% CO2, and 95% humidity as described (33). These cell lines expressing TLRs contain the CD25 gene under the control of E-selectin promoter, which contains an NF-κB-binding site. Thus, CD25 expression is dependent upon NF-κB activation (33, 34). Plates were plated (1 × 10^6 cells/well) in 24-well tissue culture dishes, cultured for 24 h, and stimulated with live C. burnetii phase II at a ratio of 10, 100, or 1000 bacteria per cell; 10, 100, 1000, or 1000 ng/ml of purified LPS; or 82 nmol (in regard to Lipid A). Controls included UV-killed E. coli (HB101) and Staphylococcus aureus (ATCC 12692), and LPS and lipid A extracted from E. coli and Bordetella pertussis. After 18 h stimulation, cells were stained with (R)-phycocerythrin-labeled anti-CD25 (mouse monoclonal antibody to human CD25, (R)-phycocerythrin conjugate; CALTAG Laboratories, Burlingame, CA) 1:200 and examined by flow cytometry (BD Biosciences) as described previously (35).

**Mice and Primary Macrophages Culture—** C57BL/6 wild-type mice and C. burnetii mutant (C57/HeJ) and control (C3H/HePas) mice were purchased from the University of São Paulo (Brazil), and TLR2-null mice were provided by Shizuo Akira (Osaka University, Japan) and backcrossed 8 times to C57BL/6 to ensure similar genetic backgrounds. C57BL/6, used as control mice, were obtained from CEDEME/UNIFESP (São Paulo, Brazil). Bone marrow-derived macrophages were generated as described (36) from 6- to 8-week-old mice. Differentiated macrophages were counted and seeded (2 × 10^6) in either 96-well tissue culture plates (for cytokine determination) or 24-well tissue culture plates containing a glass coverslip (for infection studies). Cultures were kept at 36 °C in a 5% CO2 in RPMI supplemented with 10% of fetal bovine serum and 5% of conditioned medium derived from cultures of L929 cells. Human PBMC were isolated from heparinized blood by Ficoll-Paque gradient centrifugation as described (27). Cells were resuspended in complete RPMI supplemented with fetal bovine serum and plated (1 × 10^6 cells/well) in 96-well plates.

**Production of IL-12/TNF-α by Infected Macrophages—** PBMC were cultured and stimulated in 96-well tissue culture plates with C. burnetii phase I lipid A (0.1, 1, or 10 μg/ml), doubly extracted LPS from E. coli strain O111:B4 (0.1, 1, or 10 ng/ml), PMA (30 ng/ml), and/or E564 mouse macrophages (10 ng/ml). Supernatants were harvested for TNF-α and IL-12 (p40) determination 18 h after stimulation. Murine macrophages were infected in 96-well tissue culture plates at a multiplicity of 10, 100, or 500 bacteria per cell (in a final volume of 200 μl/well) in the presence or absence of 50 units/ml of IFN-γ (R & D Systems, Minneapolis, MN). Aliquots of the supernatant were collected 24 and 48 h after infection, respectively, for the determination of the presence of TNF-α and IL-12 (p40). Cytokines were measured by a commercially available lipid A (about 200 ng/ml).

**Determination of C. burnetii Viability and Percentage of Cells with LRVs—** The viability of C. burnetii was determined as described previously (36). Briefly, infected macrophages were submitted to hypotonic lysis with H2O, a step that did not reduce the bacterial infectivity. Lysates were sonicated, diluted, and then used to infect monolayers of γ-irradiated (1000 rads) Vero cells to block cell multiplication. Irradiated Vero cells were cultured at a density of 2 × 10^4 cells/well in 24-well tissue culture plates seeded by DAPI 4 days after infection. An epifluorescence microscope equipped with a ×40 objective was used to score the percentage of infected cells. Dilutions chosen for counting contained in the range of 10–50% infected cells. The percentage of cells with LRVs was determined as described (36), using a ×40 objective in an inverted microscope to score the presence or absence of large C. burnetii vacuoles. Approximately 500 cells per well were counted, and the percentage of cells containing LRVs was recorded. Finally, infected cultures were scored for either LRV formation or viability determination.

**Confocal Microscopy, Image Acquisition, and Determination of C. burnetii Load in LRVs—** Images of fixed and DAPI-stained cells (stained for 15 min with 3.5 μM DAPI) were acquired in a Bio-Rad 1024UV confocal system as described (27). MetaMorph (Universal Imaging Corp.) version 3.5 was used for image processing. Polygons were drawn onto digitized images of infected cells, and the relative percentage of infected cells was determined as described (27). Images of fixed and DAPI-stained cells were obtained from cultures of L929 cells. Human PBMC were isolated from heparinized blood by Ficoll-Paque gradient centrifugation as described (27). Cells were resuspended in complete RPMI supplemented with fetal bovine serum and plated (1 × 10^6 cells/well) in 96-well plates.
fluorescence intensity of DAPI-stained bacteria within the circumscribed areas was determined. Under the measurement conditions used, it was shown that the fluorescence intensity within each polygon is proportional to the bacterial load in the region measured (27). Between 60 and 90 vacuoles were measured in each of triplicate coverslips.

Electrospray Ionization-Mass Spectrometry—Lipid A species were analyzed using an electrospray ionization-trap-mass spectrometer (ESI-MS) (LCQ-Duo, ThermoFinnigan, San Jose, CA). Samples were diluted in chloroform/methanol (1:1, v/v), containing 10 mM ammonium acetate (CM/AA), and introduced into the electrospray source through a fused silica capillary (50-μm internal diameter), using a microinfusion pump (Harvard Apparatus) at a flow rate of 5–10 μl/min, or through a 20-μl loop of the ESI-MS instrument with the assistance of a solvent delivery system (Omnifit, UK) containing CM/AA and pressurized with N₂ (9–10 pounds/square inch). Spectra were collected in negative ion mode, using an ion source voltage of 4.2 kV and capillary voltage and temperature of 35–45 V and 200 °C, respectively. Full scans were acquired at a rate of 3 scans/s, over the mass range of 200–2000 m/z. Fragmentation analysis (ESI-MS/MS) was carried out using a relative collision energy of 20–40% (1–2 eV). Authentic lipid A preparations from *E. coli* and *Salmonella minnesota* (Sigma) were used to calibrate instrument parameters in both ESI-MS and ESI-MS/MS modes.

**FIG. 1.** *C. burnetii* phase II activates TLR2 but not TLR4. A, CHO cells expressing TLR2 (TLR2⁺), TLR4 (TLR4⁺), or neither (TLR2⁻/TLR4⁻) were either untreated (black) or exposed to live *C. burnetii*, inactivated *E. coli* (10⁷ bacteria/cell), or 5 × 10⁵ inactivated *S. aureus* per cell. CD25 expression was measured by flow cytometry 18 h after stimulation (gray line). B, fold induction of CD25 estimated from the same experiment. Shown are three different concentrations of *C. burnetii*: 10, 100, and 1000 bacteria per cell. Culture media (medium) were used as negative control. *Gray bar* (TLR2⁻/TLR4⁻); *black bar* (TLR4⁺); *open bar* (TLR2⁺). The fold increase on CD25 expression was calculated by dividing the median fluorescence from stimulated cells by the median fluorescence from unstimulated control cells. The data represent one experiment of two performed with similar results.
TLR2 Signaling during Coxiella Infection

RESULTS

C. burnetii Phase II Bacteria Activate TLR2 but Not TLR4—CHO/CD14 reporter cell lines expressing either human TLR2 or TLR4 were stimulated for 18 h with live C. burnetii phase II at a ratio of 10, 100, and 1000 bacteria per cell. Controls included UV-killed S. aureus and E. coli. The former is known to activate only TLR2, whereas the latter contains components that activate both TLR2 (such as lipopeptides and peptidoglycan) and TLR4 (lipopolysaccharide) (38, 39). NF-κB activation was assessed by measuring the expression of surface CD25 by flow cytometry (33, 34). No induction of CD25 expression was observed when cells expressing TLR4 were exposed to C. burnetii phase II. In contrast, induction of CD25 expression by TLR2-expressing cells was observed. These data indicate that live C. burnetii can activate TLR2 but not TLR4 (Fig. 1).

TLR2 IsImportant for the Production of Pro-inflammatory Cytokines by C. burnetii-infected Murine Macrophages—The experiments with CHO reporter cell lines suggested that C. burnetii stimulates TLR2 but not TLR4. To determine whether TLR2 is important for host recognition, macrophages from mice deficient for TLR2 (TLR2−/−) or TLR4 (C3H/HeJ) were infected with phase II C. burnetii, and the detection of bacteria by host cells was assessed by cytokine production. Macrophages from C3H/HePas and C3H/HeJ both produced high levels of TNF-α and IL-12p40 after C. burnetii infection (Fig. 2). In contrast, macrophages from TLR2−/− mice were severely defective in cytokine production when compared with macrophages from wild type mice (Fig. 2). Additionally, treating macrophages with IFN-γ before infection enhanced TNF-α and IL-12 production by wild type and TLR4-deficient macrophages but not by TLR2-deficient macrophages (Fig. 2, B and D). These findings indicate that TLR2 is required for the signaling pathway leading to production of pro-inflammatory cytokines by macrophages exposed to phase II C. burnetii and further suggest that TLR4 is not responding to C. burnetii.

TLR2 Responses Limit Growth of C. burnetii in Murine Macrophages—To determine the contribution of TLR2 or TLR4 on host cell defense, macrophages from C3H/HeJ, C3H/HePas, TLR2−/−, and wild type (C57BL/6) mice were infected with C. burnetii phase II and monitored for 8 days after infection. Bacterial replication was determined by measuring focus-forming units over time. Fig. 3A shows that C. burnetii multiplication in TLR4-deficient macrophages was similar to that observed in control macrophages that produce functional TLR4. By contrast, TLR2-deficient macrophages were found to be highly susceptible to C. burnetii multiplication.

We have demonstrated previously that murine macrophages have the ability to control the development of the LRVs in which C. burnetii multiplies (36, 40). Bacterial multiplication correlates with LRV formation (36, 41, 42). Thus, TLR2-deficient macrophages were examined to determine whether they contained a greater number of LRVs compared with control macrophages. Fig. 3B shows that macrophages from TLR2−/− mice had more LRVs than control macrophages infected in parallel. After 2 days of infection, more than 50% of the TLR2−/− macrophages contained LRVs, while less than 25% of the macrophages from C57BL/6 or C3H/HeJ or C3H/HePas displayed LRVs at this time. To investigate whether LRVs found in TLR2-deficient macrophages contained more C. burnetii cells, intravascular bacterial loads were measured using quantitative fluorescence in digital images of DAPI-stained cells (27). Fig. 3C shows that DAPI fluorescence of LRVs in macrophages from C3H/HePas mice did not differ from that found in TLR4-deficient macrophages. However, the DAPI intensities for LRVs found in macrophages deficient in TLR2 were significantly higher than those of LRVs from wild type macrophages (Fig. 3C). Fig. 3D shows representative fields of macrophage cultures infected for 4 days with C. burnetii phase II. Several macrophages from TLR2−/− mice display LRVs, whereas only a few macrophages from C57BL/6 mice developed LRVs.
these organelles. The images shown in Fig. 3D also highlight the higher bacterial load found in vacuoles formed in macrophages deficient in TLR2, as compared with wild type macrophages. Overall, these results show that wild type macrophages were more effective than TLR2-deficient macrophages at controlling C. burnetii multiplication both by reducing the efficiency of LRV formation and by inhibiting the rate of bacterial growth within the LRV.

Highly Purified Lipid A from C. burnetii Is a Weak TLR4 Agonist and Can Interfere with Activation of TLR4 on PBMC in Response to E. coli LPS—Because C. burnetii phase I LPS is known to differ structurally from that of phase II (6–8), TLR4 activation by purified phase I and phase II LPS was tested. Fig. 4 shows dose-dependent activation of NF-κB in cells expressing TLR2, but not functional TLR4, in response to purified C. burnetii LPS. These data demonstrated that TLR4-depend-
ent responses are not triggered by either *C. burnetii* phase I or phase II LPS. LPS preparations can contain low concentrations of highly bioactive contaminants that can be removed upon further purification (43–45). To further investigate whether LPS from *C. burnetii* could activate TLR2, highly purified lipid A from *C. burnetii* was obtained by mild acid hydrolysis of LPS, followed by extensive Folch’s partition.

NF-κB activation in cells expressing either TLR2 or TLR4 was not observed in response to lipid A from either phase I or II *C. burnetii* (Fig. 5). Controls included lipids A from *E. coli* and *B. pertussis*, both of which activated TLR4, but not TLR2 (Fig. 5). To rule out the possibility that lipid A was lost during the extensive purification, the ability of the purified lipid A preparations to antagonize activation of TLR4 by *E. coli* LPS was investigated. *C. burnetii* phase I lipid A was added to PBMC in combination with *E. coli* LPS at different concentrations. The addition of *C. burnetii* phase I lipid A did not stimulate TNF-α production by PBMC (Fig. 6); however, the addition of *E. coli* LPS resulted in production of inflammatory cytokines by these cells. When *C. burnetii* lipid A was added in combination with *E. coli* LPS, cytokine production was reduced significantly (Fig. 6). The ability of *C. burnetii* lipid A to function antagonis-
tically was evident when 1 ng/ml of *E. coli* LPS was used together with 0.1, 1, or 10 μg/ml of lipid A or when 10 ng/ml of *E. coli* LPS was used with 1 or 10 μg/ml of *C. burnetii* lipid A (Fig. 6). Similar competitive inhibition of LPS activation of TLR4-dependent responses was described to other natural lipids, such as *Rhodobacter sphaeroides* lipid A and lipid IVa (37). A more potent antagonistic activity was observed with the synthetic compound E5564 (Fig. 6), which is a known antagonist of TLR4 activation by *E. coli* endotoxin (46). To make certain that *C. burnetii* lipid A was interfering with TLR4 activation and not a downstream signaling pathway, monocytes were treated with 30 ng/ml of PMA in combination with 0.1, 1, or 10 μg/ml of *C. burnetii* lipid A. These data show that *C. burnetii* lipid A did not interfere with cytokine production by human monocytes in response to PMA. These results indicate that *C. burnetii* lipid A is interfering with TLR4 activation during Coxiella infection.

### Fig. 5. TLR2 and TLR4 are unresponsive to highly purified lipid A from *C. burnetii* phase I or II.

A. CHO cells expressing TLR2 (TLR2+), TLR4 (TLR4+), or neither (TLR2-/TLR4-) were either untreated (black) or exposed to 200 ng/ml of lipid A from *E. coli* (lipid A *E. coli*), *B. pertussis* (lipid A *B. pertussis*); *C. burnetii* phase I (lipid A phase I) or phase II (lipid A phase II). CD25 expression was measured by flow cytometry 18 h after stimulation (gray line). B, fold induction of CD25 estimated from the same experiment. Culture media (medium) were used as negative control. Gray bar (TLR2-/TLR4-); black bar (TLR4+); open bar (TLR2+). The fold increase on expression of CD25 was calculated as described in Fig. 1. The data presented are one experiment of three performed with similar results.
specifically and does not have a general effect on NF-κB signaling.

Analysis of Lipid A from C. burnetii Phase I and II LPS—Our results indicate that TLR4 responds poorly to LPS from C. burnetii. To further investigate why C. burnetii is a poor activator of TLR4, both phase I and phase II C. burnetii lipid A were analyzed by mass spectrometry (Figs. 7 and 8). Phase I and phase II lipid A preparations gave very similar spectrum profiles, depicting a series of at least 8 singly charged ([M − H]−) ion species between m/z 1364 and 1464, which were separated from each other by 14 Da (Fig. 7, A and B). The three major ion species observed at m/z 1378, 1392, and 1406 were further analyzed by ESI-MS/MS, giving rise to almost identical fragmentation spectra as we compared the two lipid A moieties (Fig. 7, C–H). The ion species at m/z 1406 and 1392 lost a 257-Da fragment, giving origin to a major daughter ion species at m/z 1149 and 1135, respectively, most probably generated by the loss of a C16:0 fatty acid chain (255 Da), ester-linked to the C-2 of the GlcN II residue, plus 2 H-protons (2 Da) from C-3 and C-4 of the hexosamine ring (47). The ion species at m/z 1378 lost a 243-Da fragment, giving rise to a major daughter ion species at m/z 1135, most likely originated by the loss of a C15:0 fatty acid chain (241 Da), probably ester-linked to the C-2 of the GlcN I residue, plus 2 protons from this hexosamine residue. Several minor ion species, 14-Da apart from each other, were observed between the parent ion species (i.e. m/z 1406, 1392, and 1378) and the major daughter ion species at m/z 1149 and 1135. These ion species most likely represent the substantial heterogeneity in the fatty acid substitution at the C-2 of GlcN I residue, as noted previously by Toman et al. (48). Other major daughter ion species at m/z 713, 695, 681, 515, 497, and 483 and several other minor ion species between m/z 727 and 437 were observed in all spectra. These ions are almost certain to be fragments containing one or two fatty acid chains, one GlcN residue, and one phosphate group, generated by collision-induced dissociation of the glycosidic linkage between the two GlcN residues (26). Most interestingly, the lack of any major ion species between m/z 1120 and 727 strongly indicates that there is no ester-linked fatty chain at C-4 of the GlcN II, in contrast to that described previously by Toman et al. (48).

According to the fragmentation profile proposed by Chan and Reid (47), this fatty acid chain would be certainly released by collision-induced dissociation. Therefore, it is likely that there might be a 3-acyloxyacyl chain attached to the amide-linked hydroxylated fatty acid at the C-2 of the GlcN II residue. Wollenweber et al. (49) have reported previously the existence of more than 50 distinct acyloxyacyl chains attached to the amide-linked fatty acids. Taken together, our ESI-MS/MS data indicate that the basic structure of the lipid A moieties isolated from LPS of C. burnetii strain Nine Mile (phases I and II) might be slightly different from that proposed by Toman et al. (48) for the lipid A moieties of LPS from strains Henzerling and S (Fig. 8). It is worth pointing out that the proposed structure lacks the phosphate group at C-1 of GlcN I residue, most probably released by the mild acid hydrolysis procedure used here. Further careful detailed analyses by gas chromatography-mass spectrometry and NMR are required to determine the definitive structure of the lipid A species studied in this work.

DISCUSSION

It was determined that murine macrophages deficient for TLR2 were markedly susceptible to infection with the intracellular bacterium C. burnetii phase II. These findings correlate with the impaired production of inflammatory cytokines by macrophages from TLR2−/− mice after C. burnetii stimulation. The fact that TLR2-deficient macrophages did not produce IL-12 and TNF-α, even with addition of exogenous IFN-γ, highlights the fundamental role of TLR2 in C. burnetii recognition. These findings were also corroborated by experiments using CHO reporter cell lines. TLR4-specific responses were not detected in response to C. burnetii. Macrophages from TLR4-deficient mice responded similarly to C. burnetii as wild type control cells, indicating that TLR4 does not mediate recognition of C. burnetii. In accordance with our findings, Honstettre and colleagues (24) showed that TLR4 is not important in controlling C. burnetii infection either in vivo or in vitro. However, a role for TLR4 in macrophage phagocytosis, in vivo cytokine production, and granuloma formation in response to C. burnetii infection was proposed (24). It should be noted that unlike this previous study, our study used clonal isolates of C. burnetii.
(phase I, clone 7 RSA493, and phase II, clone 4 RSA439), which could account for differences in observed responses. Experiments performed with a homogeneous bacterial population ensures that the LPS or lipid A molecules being displayed will be highly similar (as observed in the spectra shown in Fig. 7), which is of particular importance given that slightly different LPS structures could account for variations in TLR activation (20, 50).

*C. burnetii* has been reported to stimulate the synthesis of pro-inflammatory cytokines by macrophages and monocytes *in vitro* (51, 52). Most interestingly, the overproduction of cytokines is a clinical manifestation associated with acute Q fever (53). Although it had been shown previously that treatment of macrophages *ex vivo* with TNF-α increases host cell resistance to *C. burnetii* (54), the receptors involved in triggering cytokine production had not been elucidated. In this study, it was found that TLR2 is the major receptor that recognizes bacterial components. TLR2 was necessary for the production of inflamma-

---

**Fig. 7.** ESI-MS and ESI-MS/MS (negative-ion mode) analysis indicates that lipid A moieties isolated from LPS of *C. burnetii* phase I and phase II are similar. Lipid A samples were dissolved in CM/AA solvent mixture and analyzed as described under “Experimental Procedures.” A and B, ESI-MS (MS1) spectra; C–H, ESI-MS/MS (MS2) spectra.
structures that mimic lipid A from nonpathogenic bacteria. These synthetic molecules are not only potent inhibitors of enterobacterial LPS activation of TLR4 but, when tested with transfected cell lines, appear to inhibit TLR2 activation as well. Thus, it is possible that lipid A has the potential to interact with both TLR2 and TLR4 and to trigger biologically significant responses.

Although the ability of lipid A to activate TLR2 has been difficult to demonstrate, findings that indicate some lipid A molecules are poor activators and function as antagonists of TLR4 are less controversial (50). Our data indicate that C. burnetii lipid A falls into this category. Most interestingly, in terms of the number and size of the fatty acid chains, the structure of C. burnetii lipid A described here is similar to lipid IVa from E. coli and lipid A from R. sphaeroides (RSLA), molecules that are unable to stimulate human monocytes (37) and function as effective TLR4 inhibitors. A new paradigm seems to be emerging from this and other work: when a lipid A species fails to stimulate human TLR4, it is likely to be a TLR4 antagonist. Such observations have been made by the pharmaceutical industry, which has already developed many TLR4 inhibitors by using this knowledge.

TLR-mediated recognition of microbial determinants is critical for the establishment of an acquired immune response that can contain and clear bacterial infections (55). An important aspect of the TLR family members is their ability to recognize multiple pathogen-associated molecular patterns present in the same class of pathogens. For example, Gram-negative bacteria are recognized by TLR2 in combination with either TLR1 or TLR6 (lipoproteins/lipopeptides, lipoteichoic acid, and peptidoglycan), TLR4 (LPS), TLR5 (flagellin), and TLR9 (CpG DNA). The capacity of TLRs to recognize multiple targets in a single infectious agent means that the contribution of a single TLR in pathogen detection may not be essential for protection. It is likely that highly adapted mammalian pathogens, such as C. burnetii, have strategies to escape host immune detection, data presented here support this hypothesis by showing that C. burnetii can avoid detection by TLR4 but is still recognized by TLR2. Although TLR2 detection of C. burnetii by macrophages ex vivo resulted in the production of inflammatory cytokines and host cell resistance, it remains possible that the evasion of TLR4-mediated responses may facilitate infection and replication in vivo.

Stimulation of different TLRs can have a synergistic effect on innate immune responses, helping to shape a response that is appropriate for the type of organism detected. It remains to be determined whether other TLRs are involved in C. burnetii recognition; however, our data suggest that detection is primarily a TLR2-dependent event. A severe defect in cytokine production after C. burnetii infection was observed for macrophages lacking only TLR2, suggesting that detection by other TLRs is insufficient for a robust host response. A lack of response by other TLRs may be related to the lack of targets and the intracellular lifecycle of C. burnetii. For instance, C. burnetii degradation leading to the release and TLR9-mediated detection of CpG DNA is less likely than for other pathogens, given that these bacteria thrive in an acidic phagolysosomal vacuole and are highly resistant to the hydrolytic enzymes present in this organelle (56). Furthermore, C. burnetii do not have the genes necessary for the production of flagella, making recognition by TLR5 unlikely (57). Based on these data, we speculate that C. burnetii evasion of host immunity during initial stages of infection and disease development may be
related to a lack of integration of multiple TLR-mediated signaling pathways. This could facilitate the high infectivity of *C. burnetii*, where it has been argued that a single organism is sufficient to cause disease. Future studies aimed at defining biochemical interactions between *C. burnetii* molecules and specific TLRs may provide new insights into the complex mechanisms of pathogenesis and consequently aid in the development of a vaccine that effectively prevents Q fever.

Acknowledgments—We thank Michel Rabinovitch (Federal University of São Paulo, Brazil) for the constructive discussions and suggestions in experimental approach and to Shizuo Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) for providing the TLR2−/− mice.

REFERENCES

1. Baca, O. G., Li, Y. P., and Kumar, H. (1994) *Trends Microbiol.* 2, 476–480
2. Maurin, M., Benoliel, A. M., Bongrand, P., and Raoult, D. (1992) *Infect. Immun.* 60, 5013–5016
3. Beron, W., Gutierrez, M., Rabinovitch, M., and Colombo, M. I. (2002) *Infect. Immun.* 70, 5816–5821
4. Heinzen, R. A., Hackstadt, T., and Samuel, J. E. (1999) *Trends Microbiol.* 7, 149–154
5. Baca, O. G., and Paretsky, D. (1983) *Microbiol. Rev.* 46, 127–149
6. Hoover, T. A., Culp, D. W., Vodkin, M. H., Williams, J. C., and Thompson, H. A. (2002) *Infect. Immun.* 70, 6736–6733
7. Amano, K., and Williams, J. C. (1984) *J. Bacteriol.* 160, 894–1002
8. Schramek, S., and Mayer, H. (1982) *Infect. Immun.* 38, 53–57
9. Maurin, M., and Raoult, D. (1999) *Clin. Microbiol. Rev.* 12, 518–553
10. Norlander, L. (2000) *Microbes Infect.* 2, 417–424
11. Jackson, C. A., Jr., and Medzhitov, R. (2002) *Curr. Opin. Immunol.* 14, 137–142
12. Marrie, T. J. (2004) *Infect. Immun.* 72, 416–423
13. Janeway, C. A., Jr., and Medzhitov, R. (2002) *Curr. Opin. Infect. Dis.* 15, 5–11
14. Muroi, M., Ohnishi, T., Azumi-Mayuzumi, S., and Tanamoto, K. (2003) *Infect. Immun.* 71, 3221–3226
15. Campos, M. A., Almeida, I. C., Takeuchi, O., Akira, S., Paganini, E., Procopio, D. O., Travassos, L. R., Smith, J. A., Golenbock, D. T., and Ghosh, S. (2004) *Infect. Immun.* 72, 416–423
16. Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Monja, Y., Miyake, K., and Akira, S. (2002) *J. Immunol.* 169, 1325–1332
17. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids* 5, 1–11
18. Folch, J., Lees, M., and Stanley, G. H. S. (1957) *J. Biol. Chem.* 226, 353–367
19. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) *Lipids* 5, 1–11
20. Darveau, R. P., Pham, T. T., Lemley, K., Reife, R. A., Bainbridge, B. W., Coats, H. M., Lee, K. H., Carty, H. A., Scanlan, D., Heinzen, R. A., Thompson, H. A., Samuel, J. E., Fraser, C. M., and Heidelberg, J. F. (2003) *Proc. Natl. Acad. Sci. U.S.A.* 100, 5441–5446
21. Takeda, K., Kaisho, T., and Akira, S. (2003) *Annu. Rev. Immunol.* 21, 335–376
22. Zamboni, D. S., Freymuller, E., Mortara, R. A., and Rabinovitch, M. (2002) *Microbes Infect.* 4, 591–598
23. Westphal, O., and Jann, K. (1965) *Methods Carbohydr. Chem.* 5, 83–91
24. Schramek, S., and Mayer, H. (1982) *Infect. Immun.* 38, 53–57
25. Williams, J. C., and Waag, D. M. (1991) in *The Biology of Coxiella burnetii*. (Williams, J. C., and Thompson, H. A., eds) pp. 175–222, CRC Press, Inc., Boca Raton, FL
26. Tanum, R., Giaridel, P., Andra, J., Slaka, R., Hussein, A., Koch, M. H., and Brandenburg, R. (2004) *BMC Biochem.* 5, 1–28
27. Zamboni, D. S., Mortara, R. A., and Raubinovitch, M. (2001) *J. Microbiol. Methods* 43, 223–232
28. Samuel, J. E., Frazier, M. E., and Mallavia, L. P. (1985) *Infect. Immun.* 49, 775–779
29. Tujulin, E., Lilliehook, B., Macellaro, A., Sjostedt, A., and Norlander, L. (1999) *Microbes Infect.* 2, 417–424
30. Zamboni, D. S., Pham, T. T., Lemly, K., Reife, R. A., Bainbridge, B. W., Coats, H. M., Lee, K. H., Carty, H. A., Scanlan, D., Heinzen, R. A., Thompson, H. A., Samuel, J. E., Fraser, C. M., and Heidelberg, J. F. (2003) *Proc. Natl. Acad. Sci. U.S.A.* 100, 5441–5446
31. Almeida, I. C., Camargo, M. M., Procopio, D. O., Silva, L. S., Meltzer, A., Travassos, L. R., Gazzinelli, R. T., and Ferguson, M. A. (2000) *EMBO J.* 19, 1476–1485
32. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Ward, D. C., Qureshi, N., Michalek, S. M., and Vogel, S. (2001) *Infect. Immun.* 69, 23–26
33. Westphal, O., and Jann, K. (1965) *Methods Carbohydr. Chem.* 5, 83–91
34. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Adem, A. (2001) *Nature* 410, 1099–1103
35. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Adem, A. (2001) *Nature* 410, 1099–1103
Stimulation of Toll-like Receptor 2 by *Coxiella burnetii* Is Required for Macrophage Production of Pro-inflammatory Cytokines and Resistance to Infection

Dario S. Zamboni, Marco A. Campos, Ana C. T. Torrecilhas, Kati Kiss, James E. Samuel, Douglas T. Golenbock, Fanny N. Lauw, Craig R. Roy, Igor C. Almeida and Ricardo T. Gazzinelli

*J. Biol. Chem.* 2004, 279:54405-54415.
doi: 10.1074/jbc.M410340200 originally published online October 13, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410340200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 55 references, 29 of which can be accessed free at http://www.jbc.org/content/279/52/54405.full.html#ref-list-1