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Lipopolysaccharide from Coxiella burnetii Is Involved in Bacterial Phagocytosis, Filamentous Actin Reorganization, and Inflammatory Responses through Toll-Like Receptor 4

Amélie Honstettre,* Eric Ghigo,† Alix Moynault,* Christian Capo,* Rudolf Toman,‡ Shizuo Akira,‡ Osamu Takeuchi,‡ Hubert Lepidi,* Didier Raoult,* and Jean-Louis Mege2*

The role of Toll-like receptors (TLRs) in the recognition of extracellular and facultative intracellular bacteria by the innate immune system has been extensively studied, but their role in the recognition of obligate intracellular organisms remains unknown. Coxiella burnetii, the agent of Q fever, is an obligate intracellular bacterium that specifically inhabits monocytes/macrophages. We showed in this study that C. burnetii LPS is involved in the uptake of virulent organisms by macrophages but not in that of avirulent variants. The uptake of virulent organisms was dependent on TLR4 because it was reduced in macrophages from TLR4−/− mice. In addition, LPS was responsible for filamentous actin reorganization induced by virulent C. burnetii, which was prevented in TLR4/H11546−/− macrophages. In contrast, the intracellular fate of C. burnetii was not affected in TLR4/H9253−/− macrophages, suggesting that TLR4 does not control the maturation of C. burnetii phagosome and the microbicidal activity of macrophages. These results are consistent with in vivo experiments because the pattern of tissue infection and the clearance of C. burnetii were similar in wild-type and TLR4/H11546−/− mice. We also showed that the number of granulomas was decreased in the liver of infected TLR4/H11546−/− mice, and the formation of splenic granulomas was only transient. The impaired formation of granulomas was associated with decreased production of IFN-γ and TNF. Taken together, these results demonstrate that TLR4 controls early events of C. burnetii infection such as macrophage phagocytosis, granuloma formation, and cytokine production.

Coxiella burnetii, the etiologic agent of Q fever, is an obligate Gram-negative bacterium that inhabits monocytes/macrophages (4). Only LPS has been associated with bacterial virulence. Indeed, antigenic variation similar to smooth-to-rough variation of enterobacteria has been described in C. burnetii: bacteria in phase I (virulent organisms) express a smooth-type LPS (phase I LPS) whereas bacteria in phase II (avirulent organisms) exhibit a rough-type LPS (phase II LPS) (5). Phase II LPS is truncated and lacks the branched-chain sugars virenose and dihydroxyhydroxystreptose present in phase I LPS (6). The antigenic phase transition of LPS is associated with a chromosomal deletion (7); this concerns a large group of LPS biosynthetic genes arranged in O-Ag cluster including genes involved in virenose synthesis (8). The role of LPS in the pathogenicity of C. burnetii is poorly understood. It plays a role in bacterial immunogenicity and induces a strong Ab response (9). Although C. burnetii LPS is considered as poorly endotoxic in contrast to LPS from enterobacteria (10), it induces the production of inflammatory cytokines in murine and human macrophages (11, 12). We have previously found that TNF production by human macrophages is differentially induced by phase I and phase II LPSs (12).

C. burnetii virulence depends on the ability of organisms to enter macrophages and to escape from their microbicidal activity. Indeed, virulent organisms are poorly internalized and survive in human monocytes, whereas avirulent variants are efficiently internalized but are eliminated. The uptake of virulent C. burnetii depends on αβ3 integrin whereas that of avirulent bacteria requires αβ3 integrin and CR3 (αMβ2 integrin) (13). The control of C. burnetii phagocytosis results from the inappropriate activation of host cells. Virulent organisms, but not avirulent organisms, stimulate the activation of Lyn and Hck, two src-related protein tyrosine kinases. Their activation leads to actin cytoskeleton reorganization involved in the impairment of phagocytosis of virulent...
C. burnetii (14, 15). We have recently demonstrated that the survival of virulent C. burnetii inside monocytes is based on the impaired fusion of bacterial phagosomes with lysosomes whereas phagosomes containing avirulent organisms undergo complete phagosome-lysosome fusion (16).

In this study, we investigated the role of bacterial LPS and TLR4 in C. burnetii infection in vitro and in vivo. We showed that phase I LPS, but not phase II LPS, was involved in the phagocytosis of organisms by macrophages through TLR4. In addition, phase I LPS induced filamentous (F)-actin reorganization, which was suppressed in TLR4−/− macrophages. In contrast, the interaction of C. burnetii with TLR4 did not influence the intracellular survival of bacteria in macrophages and bacterial clearance in vivo. TLR4 was involved in the formation of granulomas and the production of inflammatory cytokines in C. burnetii-infected mice. Although TLR4 is dispensable for late control of C. burnetii infection, it controls the early events of C. burnetii infection including macrophage phagocytosis, granuloma formation, and cytokine production.

Materials and Methods

Mice, cells, and bacteria

TLR4−/− mice were generated on a mixed 129/Ola × C57BL/6 background and backcrossed to C57BL/6 for seven generations as described elsewhere (17). C57BL/6 mice (Charles River Breeding Laboratories, L’Arbresle, France) were used as control mice expressing TLR4 (wild-type, wt). C3H/HeN mice (that express functional TLR4) and C3H/HeJ mice (that do not express functional TLR4) were purchased from Charles River Breeding Laboratories. TLR2−/− mice were generated on C57BL/6 background as described elsewhere (17). The human myelomonocytic cell line THP-1 was cultured in RPMI 1640 containing 25 mM HEPES, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Eryng, France) for biweekly passages (15). Murine macrophages were recovered by washing peritoneal cavity with ice-cold HBSS. They were obtained by adherence after a 2-h incubation and washing with HBSS to remove nonadherent cells. Virulent C. burnetii organisms (Nine Mile strain) were recovered from mouse spleens at 10 days postinfection, and liver, spleen, heart, lungs, and mesenteric lymph nodes were excised, and blood samples were collected for serology. The organs, fixated in 10% neutral-buffered formalin, were sectioned and embedded in paraffin. For each tissue specimen, serial 5-μm thick sections were obtained to perform H&E staining and immunohistochemical investigations. The granuloma expression was assessed by light microscopy. Granulomas were defined as collections of 10 or more macrophages and lymphocytes within the organs. The number of granulomas was determined after whole examination of at least three tissue sections of each organ. The detection of C. burnetii organisms in tissues was performed by immunohistochemistry on serial deparaffinized sections of all organs as previously described (21). Briefly, each tissue section was incubated with anti-C. burnetii rabbit Abs (at 1/2000 dilution) or normal rabbit serum as control. Immunodetection was performed with biotinylated anti-rabbit Abs and peroxidase-labeled streptavidin (Zymed, Claymore, France). Sections were then incubated with 0.05% diaminobenzidine as substrate. After washing, slides were counterstained with Mayer’s hematoxylin for 5 min and the bacteria were visualized in tissues as precipitation products. The whole number of bacteria detected within the granulomas by light microscopy was determined for each tissue section.

For the follow-up of the infection, adherent macrophages were incubated with C. burnetii (200:1 bacterium to cell ratio) for 4 h at 37°C. They were then washed to remove free bacteria; this time point was designated as day 0. Infected macrophages were cultured for 6 days, and bacteria were revealed by indirect immunofluorescence as previously described. Results are expressed as infection index that assesses the number of bacteria per positive cell and the percentage of positive cells × 100.

F-actin analysis by laser scanning confocal fluorescence microscopy

The intracellular distribution of F-actin was determined as follows: THP-1 cells (5 × 105 cells/assay) or adherent macrophages (2 × 105 cells/assay) were stimulated with C. burnetii (200:1 bacterium to cell ratio) or C. burnetii LPS in the presence and the absence of polymyxin B, and fixed with 4% paraformaldehyde. After permeabilization with 0.1 mg/ml LPC in HBSS, cells were incubated with 10 μM bodipy phallacidin (Molecular Probes) for 20 min. The specimens were examined with a laser scanning confocal fluorescence microscope (TCS 4D; Leica, Heidelberg, Germany) equipped with a 100× (NA 1.4) oil immersion lens, as previously described (14). Serial optical sections of images were collected at 0.5 μm intervals and analyzed with Adobe Photoshop V5.5 (Adobe Systems, Mountain View, CA).

Intracellular traffic of C. burnetii

Adherent murine macrophages (2 × 105 cells/assay) were incubated with C. burnetii (200:1 bacterium to cell ratio) for different periods of time. After being washed, cells were fixed with 4% paraformaldehyde, and free aldehydes were quenched with 0.5 M ammonium chloride, as previously described (21). Cells were permeabilized by 0.1% saponin (Sigma-Aldrich) in PBS containing 10% horse serum (Invitrogen) for 30 min and washed. They were then incubated for 30 min with PBS containing 0.1% saponin, 5% horse serum, 1/2000 dilution of human Abs to C. burnetii, and rabbit Abs specific for lysosome-associated membrane protein (Lamp)-1 or cathepsin D (1/500 and 1/500 dilutions, respectively; BD Transduction Laboratories, Lexington, KY). After washing, cells were incubated with 1/100 dilution of Texas Red-conjugated anti-human IgG Abs and FITC-conjugated anti-rabbit IgG Abs (Beckman Coulter) for 20 min. Thereafter, cells were washed, mounted with Mowiol (Calbiochem, San Diego, CA), and stored at 4°C until examination. The colocalization of organisms with intracellular markers was examined with a laser scanning confocal fluorescence microscope equipped with suitable filters, as described elsewhere (16). Briefly, optical sections of images (1 μm) were analyzed using Adobe Photoshop V5.5. Vacuoles containing C. burnetii were scored as positive for soluble cathepsin D when fluorescence was observed in the phagosome lumen; for Lamp-1, a membrane marker of late endosomes, vacuoles were scored as positive when a fluorescence ring surrounded organs. Approximately 30 vacuoles containing C. burnetii were scored per coverslip, and at least three distinct experiments were performed per condition. Results are expressed as the percentage of phagosomes that colocalized with intracellular markers.

Determination of in vivo infection

TLR4−/− and wt mice, aged 5–6 wk, were challenged i.p. with 5 × 109 virulent C. burnetii organisms. They were sacrificed 7, 14, and 21 days after infection, and liver, spleen, heart, lungs, and mesenteric lymph nodes were excised, and blood samples were collected for serology. The organs, fixated in 10% neutral-buffered formalin, were sectioned and embedded in paraaffin. For each tissue specimen, serial 5-μm thick sections were obtained to perform H&E staining and immunohistochemical investigations. The granuloma expression was assessed by light microscopy. Granulomas were defined as collections of 10 or more macrophages and lymphocytes within the organs. The number of granulomas was determined after whole examination of at least three tissue sections of each organ. The detection of C. burnetii organisms in tissues was performed by immunohistochemistry on serial deparaffinized sections of all organs as previously described (22). Briefly, each tissue section was incubated with anti-C. burnetii rabbit Abs (at 1/2000 dilution) or normal rabbit serum as control. Immunodetection was performed with biotinylated anti-rabbit Abs and peroxidase-labeled streptavidin (Zymed, Claymore, France). Sections were then incubated with 0.05% diaminobenzidine as substrate. After washing, slides were counterstained with Mayer’s hematoxylin for 5 min and the bacteria were visualized in tissues as precipitation products. The whole number of bacteria detected within the granulomas by light microscopy was determined for each tissue section.
mounted on a Zeiss Axiophot/DMX1200 microscope (Zeiss, Rueil Malmaison, France). Histological images were digitized with an automated image analysis system (SAMBA Technologies, Alcatel TITN, Grenoble, France). For each study, a specific interactive program providing control of analysis was developed to measure tissue section surface on each slide. The number of granulomas and organisms within each granuloma was expressed per tissue in square millimeters.

The presence of Abs to C. burnetii in serum from wt and TLR4−/− mice was determined by microimmunofluorescence, as described elsewhere (24).

### Cytokine determination

Splenocytes and adherent macrophages from wt and TLR4−/− mice were incubated in RPMI 1640 containing 25 mM HEPES, 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). All media were checked for the absence of endotoxins with Limulus amebocyte lysate (Cambrex Bioscience, Emerainville, France). Splenocytes (2 × 10^6 cells in 1 ml) were incubated in flat-bottom 24-well culture plates (Nunc, PolyLabo, Strasbourg, France) with or without heat-inactivated C. burnetii (10 organisms per cell) for 24 h at 37°C. Once collected, supernatants were stored at −80°C until IFN-γ determination. Adherent macrophages (10^6 cells/assay) were stimulated by heat-inactivated C. burnetii (10 organisms per cell) for 24 h at 37°C, and cell supernatants were tested for the presence of TNF and IL-10. The three cytokines were measured by immunoassays. IFN-γ (detection limit: 10 pg/ml) and IL-10 (detection limit: 12 pg/ml) assays were provided by Endogen (BioAdvance, Emerainville, France). TNF (detection limit: 5 pg/ml) assay was from R&D Systems (Abingdon, U.K.). The intra- and interspecific coefficients of variation were <10%.

### Statistical analysis

Results, given as the mean ± SD, were compared with Student’s t test. Differences were considered significant at p < 0.05.

### Results

**LPS from virulent C. burnetii is involved in bacterial uptake through TLR4**

As virulent and avirulent C. burnetii organisms are differentially internalized by monocytes and exhibit distinct LPS structures, we investigated the role of LPS in phagocytosis by incubating THP-1 monocytes with polymyxin B, known to interfere with LPS binding. In the absence of serum, polymyxin B (at 5 µg/ml) decreased the uptake of virulent C. burnetii by 35% after 2 h (p < 0.03) and by 50% after 4 h (p < 0.02), but it had no effect on the uptake of avirulent organisms (Table 1). In the presence of AB serum as a source of LPS-binding protein, the uptake of virulent and avirulent C. burnetii was higher than in its absence. Again, polymyxin B inhibited the internalization of virulent organisms by 55% after 2 h (p < 0.01) and by 60% after 4 h (p < 0.02), without affecting the uptake of avirulent organisms. The role of LPS in C. burnetii uptake was not restricted to THP-1 monocytes because polymyxin B inhibited the uptake of virulent C. burnetii by monocytes isolated from peripheral blood to a similar extent (data not shown). As TLR4 is involved in the recognition of several species of LPS (25), we investigated the role of TLR4 in the uptake of C. burnetii using peritoneal macrophages from wt and TLR4−/− mice (Fig. 1). The phagocytosis of virulent organisms by macrophages from wt mice was lower than that of avirulent organisms, as described for THP-1 cells. The uptake of virulent C. burnetii was decreased by ~40% in macrophages from TLR4−/− mice with or without addition of mouse serum (p < 0.05). The decrease in uptake of virulent C. burnetii was similar in macrophages from TLR4−/− mice and in THP-1 monocytes in the presence of polymyxin B (compare Fig. 1 and Table 1). In contrast, the uptake of avirulent organisms was similar in wt and TLR4−/− macrophages (Fig. 1). C. burnetii phagocytosis was also studied in C3H/HeJ mice that do not express functional TLR4. The uptake of virulent C. burnetii by peritoneal macrophages was decreased by 45% in C3H/HeJ mice compared with C3H/HeN mice whereas the uptake of avirulent organisms was not affected by the lack of functional TLR4. Hence, the phagocytosis of virulent C. burnetii by macrophages involves bacterial LPS and TLR4, in contrast to that of avirulent organisms.

### Table I. Effect of polymyxin B on C. burnetii phagocytosis

<table>
<thead>
<tr>
<th>Polymyxin B (h)</th>
<th>Without AB Serum</th>
<th>With AB Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulent C. burnetii (2)</td>
<td>60 ± 7</td>
<td>39 ± 3*</td>
</tr>
<tr>
<td>Virulent C. burnetii (4)</td>
<td>124 ± 10</td>
<td>62 ± 4**</td>
</tr>
<tr>
<td>Avirulent C. burnetii (2)</td>
<td>179 ± 27</td>
<td>216 ± 42</td>
</tr>
<tr>
<td>Avirulent C. burnetii (4)</td>
<td>295 ± 39</td>
<td>354 ± 35</td>
</tr>
</tbody>
</table>

a THP-1 monocytes (5 × 10^6 cells/assay) were pretreated with polymyxin B (5 µg/ml) and then incubated with C. burnetii (200:1 bacterium to cell ratio) in the presence or absence of 10% human AB serum for 2 and 4 h. Bacteria were detected by immunofluorescence. Results are expressed as phagocytosis index and are the mean ± SE of five experiments.

* p < 0.03, ** p < 0.02, *** p < 0.01, which represent the comparison of C. burnetii uptake by THP-1 cells in the presence and the absence of polymyxin B.
LPS from virulent C. burnetii stimulates F-actin reorganization through TLR4

As the phagocytosis of virulent C. burnetii is associated with the formation of pseudopodal extensions and polarized distribution of F-actin (14), we investigated the involvement of bacterial LPS and TLR4 in cytoskeleton reorganization. In macrophages from wt mice, virulent C. burnetii induced cell spreading and the formation of polarized filopodia and lamellipodia. F-actin was concentrated beneath filopodia and lamellipodia and as spots in cytoplasmic areas. In contrast, avirulent variants of C. burnetii had no effect on F-actin organization (Fig. 2A). Phase I LPS reproduced C. burnetii-induced morphological changes of macrophages consisting of cell spreading, filopodia, polarized lamellipodia and cytoplasmic spots of F-actin (Fig. 2A). After 10 min of stimulation with 1 µg/ml phase I LPS, ~80% of cells exhibited filopodia, and the percentage of cells with filopodia decreased thereafter (Fig. 2B). Filopodia were detected in response to 0.25 µg/ml phase I LPS and the number of cells with filopodia became maximum with 1 µg/ml phase I LPS (Fig. 2C). In contrast to phase I LPS, phase II LPS did not stimulate the formation of filopodia and F-actin reorganization (Fig. 2A) whatever the time of stimulation and the dose of LPS. In macrophages from TLR4−/− mice, the reorganization of F-actin and the formation of filopodia stimulated by virulent C. burnetii and phase I LPS were prevented (Fig. 2). Similarly, the cytoskeleton reorganization induced by virulent C. burnetii was decreased by 75% in macrophages from C3H/HeN mice compared with macrophages from C3H/HeJ mice. Hence, C. burnetii-stimulated morphological changes and F-actin reorganization require bacterial LPS and TLR4.

TLR4 is not involved in intracellular survival of C. burnetii

As the interaction of virulent C. burnetii with TLR4 is critical for early events associated with bacterial uptake, we investigated its role in C. burnetii survival. Macrophages from wt and TLR4−/− mice were incubated with virulent and avirulent organisms for 4 h, and infected cells were cultured for 6 days. In wt macrophages infected with virulent C. burnetii, the infection index slowly decreased at day 3 postinfection and remained constant thereafter (Fig. 3A). Intracellular bacteria remained viable during the course of the experiments as assessed by measuring C. burnetii viability (data not shown). In contrast, the number of avirulent organisms markedly decreased after 3 days (80% inhibition) and the infection index was minimum at day 6 postinfection. The decreased number of bacteria corresponded to their killing, as assessed by decreased viability (data not shown). In TLR4−/− macrophages infected with virulent C. burnetii, the infection index was similar to that found in wt macrophages at days 3 and 6 postinfection, and the bacterial viability was not modified. The infection index rapidly decreased in TLR4−/− macrophages infected with avirulent variants of C. burnetii, as found in wt macrophages (Fig. 3A). Thus, the survival of virulent C. burnetii organisms in macrophages does not depend on their interaction with TLR4.

As the survival of virulent C. burnetii is associated with impaired fusion of phagosomes with lysosomes in monocytes (16), C. burnetii trafficking was investigated in TLR4−/− macrophages. For that purpose, the colocalization of C. burnetii phagosomes with Lamp-1, a marker of late endosomes-lysosomes, and cathepsin D, a lysosomal protease, was determined. In wt and TLR4−/− macrophages incubated with virulent or avirulent C. burnetii (day 0), ~70% of phagosomes colocalized with Lamp-1, which appeared as a ring surrounding the organisms. At day 2 postinfection, all phagosomes had acquired Lamp-1 (Fig. 3B). In wt and TLR4−/− macrophages, cathepsin D appeared in the lumen of 40% of phagosomes containing avirulent organisms at day 0. The percentage of phagosomes that colocalized with cathepsin D steadily increased thereafter and reached 60% at day 2 postinfection. In contrast, phagosomes containing virulent C. burnetii were unable to colocalize with cathepsin D whatever the time of postinfection in wt and TLR4−/− macrophages (Fig. 3C). Hence, TLR4 does not interfere with the trafficking of C. burnetii in murine macrophages.
Role of TLR4 in C. burnetii infection in vivo

We wondered whether the lack of TLR4 may affect the susceptibility and/or the resistance of mice toward C. burnetii. TLR4−/− and wt mice were i.p. injected with 5 × 10^5 virulent organisms, and their infection was recorded up to 21 days. Mortality or morbidity was not observed in wt and TLR4−/− mice, suggesting that mice remained resistant to C. burnetii despite the lack of TLR4. The course of infection was assessed by measuring circulating Abs. In wt mice, Abs directed against C. burnetii were detected after 7 days of infection and their titer reached a plateau after 14 and 21 days. In TLR4−/− mice, the kinetics of production of specific Abs was delayed but, after 14 days, Ab titers were similar in wt and TLR4−/− mice (Table II). The course of tissular infection was also assessed by immunodetection of C. burnetii. In wt and TLR4−/− mice, C. burnetii organisms were detected in liver and spleen, whereas lung, heart, and mesenteric lymph nodes remained devoid of organisms. The organisms were found in intracellular location as coarse or fine grains, and immunopositive cells corresponded to macrophages inside granulomas (Fig. 4A). In wt and TLR4−/− mice, the organisms were detectable after 4 days in the spleen; bacterial load reached a peak at day 7 and it decreased thereafter to become undetectable at day 21. In the liver from wt and TLR4−/− mice, C. burnetii organisms were detected at 4 days postinfection and their number steadily decreased to reach minimum amount at day 21 (Fig. 4B). Hence, bacterial clearance was independent of TLR4.

As C. burnetii organisms were detected within inflammatory granulomas, we investigated the expression of granulomas in each target organ. In wt mice, lesions appeared as aggregates mainly composed of macrophages and lymphocytes and few polymorphonuclear...
leukocytes without suppuration. The granulomatous reactions were focal and scattered throughout liver lobules, portobiliary spaces, and splenic red pulp (Fig. 5A). In the spleen of wt mice, granulomas were detected after 4 days; their number became maximized between days 7 and 14 and decreased thereafter (Fig. 5B). In the spleen from TLR4−/− mice, the number of granulomas was similar to that of wt mice after 4 and 7 days, but it was significantly lower (p < 0.02) than in wt mice at days 14 and 21 postinfection. In the liver from wt mice, the number of granuloma was high at day 4 and day 7 postinfection, and steadily decreased thereafter (Fig. 5B). In TLR4−/− mice, the number of liver granulomas was significantly lower (p < 0.02) than in wt mice at days 4 and 7 postinfection. The area of granulomas and the number of cells within the granulomas were significantly reduced in TLR4−/− mice as compared with wt mice (Fig. 5C). Taken together, these results show that TLR4 is critical for the formation of granulomas in C. burnetii-infected mice.

**Role of TLR4 in C. burnetii-stimulated production of cytokines**

The role of TLR4 in the control of granuloma formation may result from the modulation of the production of cytokines such as IFN-γ and TNF, known to be required for granuloma formation. First, splenocytes from wt and TLR4−/− mice infected by C. burnetii were incubated with or without C. burnetii, and the release of IFN-γ was assessed. In the absence of stimulation, splenocytes from uninfected mice did not release IFN-γ. IFN-γ was produced by unstimulated wt splenocytes (1165 ± 325 pg/ml) but not by unstimulated TLR4−/− splenocytes at day 7 postinfection. C. burnetii-stimulated splenocytes from uninfected wt mice produced IFN-γ to a low extent; the release of IFN-γ dramatically increased in splenocytes of wt mice infected for 7 days and remained high until day 21 (Fig. 6A). In stimulated TLR4−/− splenocytes, the release of IFN-γ was decreased by > 50% whatever the time of infection of mice, and it was almost undetectable at day 21 postinfection. Second, the production of TNF was assayed in supernatants from unstimulated macrophages. TNF was released by macrophages from uninfected wt mice (21 ± 5 pg/ml) and its amount was maximum at day 7 postinfection (71 ± 18 pg/ml); it was undetectable in TLR4−/− mice. C. burnetii-stimulated TNF production by macrophages from uninfected wt mice, and TNF production was dramatically increased in macrophages from infected mice (Fig. 6B). C. burnetii-stimulated TNF production by TLR4−/− macrophages was decreased by 50% as well in uninfected mice than as in infected mice. The partial effect of TLR4 on TNF production was specific for C. burnetii because the TNF production stimulated by LPS from E. coli was depressed by >90% whatever the time of infection of TLR4−/− mice compared with wt mice (data not shown). Third, we investigated the production of IL-10, known to down-modulate inflammatory cytokines, by peritoneal macrophages. Unstimulated macrophages from wt mice released IL-10 only at days 7 (59 ± 18 pg/ml) and 14 (38 ± 6 pg/ml) postinfection. The unstimulated release of IL-10 was undetectable in macrophages from TLR4−/− mice. In response to C. burnetii stimulation, IL-10 was produced by macrophages from uninfected and infected wt mice. In macrophages from TLR4−/− mice, C. burnetii-stimulated production was decreased by 80% at day 7 postinfection and by 50% at days 14 and 21 (Fig. 6C). Taken together, these results show that TLR4 is involved in cytokine release in C. burnetii-infected mice.

**Role of TLR2 in macrophage responses to C. burnetii**

Because TLR4 was involved in C. burnetii phagocytosis, we asked whether TLR2 was directly or indirectly involved in this response. First, we investigated the role of TLR2 in the phagocytosis of C. burnetii and the F-actin reorganization induced by virulent organisms. The uptake of virulent and avirulent C. burnetii was similar in wt and TLR2−/− mice (Fig. 7A). The lack of TLR2 did not affect the F-actin reorganization stimulated by C. burnetii (Fig. 7B). Second, we wondered whether TLR2 engagement may restore the impaired phagocytosis of C. burnetii found in TLR4−/− macrophages. For that purpose, macrophages were pretreated with PG, a specific ligand of TLR2, for 30 min and then incubated with virulent C. burnetii. The addition of PG to wt macrophages had no effect on C. burnetii phagocytosis (Fig. 7C). In contrast, the addition of PG to TLR4−/− macrophages significantly (p < 0.05) increased C. burnetii phagocytosis to the level found in wt macrophages. Taken together, these results suggest that TLR2 is not involved in C. burnetii phagocytosis but that its engagement rescued impaired phagocytosis of TLR4−/− mice.

**Discussion**

The aim of this report was to study the role of LPS in C. burnetii infection, an obligate intracellular organism. The composition of LPSs from C. burnetii is distinct from that of LPSs isolated from other Gram-negative microorganisms, which are highly endotoxic
ated protein (13). As the integrins including

sections at day 7 postinfection. The results are the mean

ulomas and the granuloma area were calculated by image analysis of liver

mice were infected by

organisms in-

C. burnetii

was not impaired in TLR4

background of mice because similar results were obtained in

because LPS from enterobacteria, known to be highly endotoxic,

induces similar reorganization of cytoskeleton in monocytes and

macrophages (29). The role of LPS in C. burnetii phagocytosis
depends on its effect on actin cytoskeleton, which controls bacte-

tial receptor redistribution and orientates C. burnetii toward the

leading edge of host cells (30).

The effect of LPS from virulent C. burnetii on bacterial phagocy-
tosis and F-actin reorganization is mediated by TLR4. To our

knowledge, this is the first demonstration of TLR4 involvement in

early cellular events associated with the phagocytosis process. The

uptake of virulent organisms was decreased in TLR4−/− macrophages as compared with wt macrophages, as observed in the pres-

cence of polymyxin B. This result did not depend on the genetic

background of mice because similar results were obtained in TLR4−/− and C3H/HeJ macrophages. It demonstrates that LPS distinct from canonical LPS isolated from enterobacteria may in-

teract with TLR4. In contrast, the uptake of avirulent variants of

C. burnetii was not impaired in TLR4−/− macrophages, suggesting that the interaction of avirulent bacteria with macrophages is independent of TLR4. This interaction did not depend on TLR2 because the phagocytosis of avirulent C. burnetii organisms was

(6). LPS from virulent C. burnetii was involved in bacterial inter-

nalization by monocytes/macrophages. Indeed, polymyxin B, a

LPS antagonist, decreased the uptake of virulent C. burnetii

by monocytes. Nevertheless, the inhibition was only partial, suggest-

ing that the role of phase I LPS in phagocytosis is complementary
to previously reported entry mechanisms. Indeed, virulent C. bur-

netii is internalized by monocytes through the engagement of α3β2

integrin whereas the uptake of avirulent organisms engages both

α3β2 integrin and CR3 through the activation of integrin-associated

protein (13). As the integrins including α3β2 integrin are un-
able to support efficient binding and internalization in resting cells without activating signals, it is likely that phase I LPS mediates a

costimulation signal enabling α3β2 integrin to acquire an active

conformation, and thus allows C. burnetii recognition and inter-

nalization. This is reminiscent of the role of LPS in β2 integrin

activation (26). LPS-mediated activation of integrins may depend

on their interaction with cytoskeleton (27, 28). We have previously

reported that virulent but not avirulent C. burnetii organisms

induce reorganization of F-actin in monocytes (15). C. burnetii-stim-

ulated reorganization of F-actin was due to bacterial LPS. The LPS

isolated from virulent C. burnetii induced the formation of filo-
odia and lamellipodia in murine macrophages, as did intact C.
burnetii organisms. This property is not specific of C. burnetii LPS
because LPS from enterobacteria, known to be highly endotoxic,
similar in TLR2−/− and wt macrophages. This finding is distinct from reports in which atypical LPS from Porphyromonas gingivalis or Leptospira interrogans are recognized by TLR2 but not by TLR4 (31, 32). Nevertheless, TLR2 may compensate the lack of TLR4 for C. burnetii phagocytosis. Indeed, PG, known to specifically interact with TLR2, increased C. burnetii phagocytosis by TLR4−/− macrophages to the level found in wt macrophages. Lipopeptide, another ligand of TLR2, was recently shown to increase phagocytosis of opsonized latex beads in neutrophils (33). We also found that C. burnetii-stimulated reorganization of F-actin in macrophages is mediated by TLR4. Indeed, in TLR4−/− macrophages, virulent organisms and phase I LPS were unable to stimulate the formation of lamellipodia and filopodia. The role of TLR4 in cytoskeleton reorganization has been previously evoked in macrophages from C3H/HeJ mice stimulated by E. coli LPS in which the disruption of microfilament network was prevented (34). The mechanisms of TLR4-mediated F-actin reorganization remains hypothetical, but it may be related to the activation of src-related kinases because it has been shown that C. burnetii-stimulated F-actin reorganization depends on src kinase activation (15).

Although initial events of C. burnetii infection of macrophages depend on TLR4, the survival of C. burnetii does not. Virulent C. burnetii survived in macrophages from wt and TLR4−/− mice, and avirulent organisms were cleared by both types of macrophages. This finding suggests that TLR4 is dispensable for C. burnetii survival. We recently showed that the survival of C. burnetii in human monocytes is associated with impaired phagosome maturation (16), but the role of TLRs in intracellular traffic of microorganisms is largely ignored. It has been only suggested that TLR2 is recruited to phagosomes containing yeasts (35) and IgG-coated E. coli (36). In murine macrophages from wt and TLR4−/− mice, phagosomes containing virulent C. burnetii did not acquire cathepsin D in contrast to phagosomes containing avirulent organisms. This extends to murine macrophages the impairment of phagosome-lysosome fusion reported in human monocytes (16). In addition, phagosomes containing virulent or avirulent organisms acquired Lamp-1, demonstrating the integrity of upstream traffic as described in human monocytes (16). Hence, the lack of TLR4 had no effect on phagosome maturation in murine macrophages infected with C. burnetii. We also found that the survival of C. burnetii in vivo does not involve TLR4. Indeed, the i.p. injection of C. burnetii into mice leads to the accumulation of bacteria in spleen and liver followed by their clearance in a similar way in wt and TLR4−/− mice. The role of TLR4 in infections caused by intracellular pathogens has been poorly documented. Hence, C3H/HeJ mice initially control Mycobacterium tuberculosis infection but they succumb later (37). In mice with TLR4 mutation, the early clearance of Ehrlichia chaffeensis is suppressed but the subsequent resistance is preserved (38). Infection with Legionella pneumophila proceeds in an identical way in TLR4−/− and wt mice (39). These results suggest that TLR4 is not critical for the clearance of intracellular microorganisms including C. burnetii.

The immune host response to C. burnetii infection depends on TLR4. Indeed, C. burnetii infection results in the formation of granulomas in spleen and liver, which has been associated with protective immune response (40). In TLR4−/− mice, the expression of granulomas in spleen was more transient than in wt mice, and the number of granulomas in liver was decreased as compared with wt mice. In addition, the cell density of granulomas was lower in infected TLR4−/− mice than in wt mice. The mechanisms of altered formation of granulomas are likely multiple. They may involve changes in granuloma cell composition or impaired cytokine production necessary to granuloma formation. Hence, in mice infected with M. tuberculosis, the lack of TLR4 is associated with increased number of granulomas and increased influx of neutrophils in granulomas (37). However, the cellular composition of granulomas in mice infected with C. burnetii was similar in TLR4−/− and wt mice. The mechanisms leading to granuloma formation involve the production of inflammatory cytokines including IFN-γ and TNF as demonstrated by knockout mice (41–43). We showed in this study that C. burnetii-stimulated production of IFN-γ by splenocytes was lower in TLR4−/− mice than in wt mice. Such results suggest that TLR4 is involved in C. burnetii-induced granuloma formation via IFN-γ production. This is distinct from Abel et al. (37) report in which M. tuberculosis-infected C3H/HeJ mice express more granuloma than C3H/HeN mice without modulation of IFN-γ production. Similarly, C. burnetii-stimulated production of TNF was down-modulated in TLR4−/− mice as compared with wt mice. This finding agrees with some previous

![FIGURE 7. TLR2 and macrophage responses to C. burnetii. A and B, Macrophages (2 × 10⁵ cells/assay) from wt and TLR2−/− mice were incubated with virulent and avirulent C. burnetii (200:1 bacterium to cell ratio) for 4 h (A) and 10 min (B). A, Bacteria were detected by immunofluorescence. The results are expressed as phagocytosis index and are the mean ± SE of three experiments. B, F-actin was labeled with 10 U/ml bodipy phallacidin, and cells were examined by laser scanning confocal microscopy. The results are expressed as the percentage of macrophages showing filopodia and represent the mean ± SE of three experiments. C, Macrophages (2 × 10⁵ cells/assay) from wt and TLR4−/− mice were pretreated by PG (50 mg/ml) for 30 min and incubated with virulent C. burnetii (200:1 bacterium to cell ratio) for 4 h at 37°C. Bacteria were detected by immunofluorescence. The results are expressed as phagocytosis index and are the mean ± SE of three experiments. *p < 0.05 represents the comparison of C. burnetii uptake by TLR4−/− macrophages in the presence and the absence of PG.](http://www.jimmunol.org/DownloadedFrom)
reports in which TNF production induced by soluble LPSs, Gram-negative bacteria and M. tuberculosis was dramatically impaired when TLR4 is lacking (37). The results concerning impaired production of IFN-γ and TNF in TLR4−/− mice are consistent with the hypothesis that the engagement of TLR4 leads to the production of type I cytokines required for protection against intracellular microorganisms, in contrast to TLR2 engagement that favors the production of type 2 cytokines (2, 44). The down-modulation of granuloma formation does not result from increased production of cytokines known to impair the production of type 1 cytokines such as IL-10. Indeed, the production of IL-10 was markedly impaired by macrophages and C. burnetii organism such as C. burnetii, as IL-10. Indeed, TLR4 was involved in the uptake of virulent organism such as C. burnetii. Indeed, TLR4 likely plays a critical role in the early responses of the host to C. burnetii infection by enabling it to develop protective immune responses, but the control of late phases of the infection requires other mechanisms.

We reported in this study that LPS from an obligate intracellular organism such as C. burnetii required TLR4 to transduce signals in host cells. Indeed, TLR4 was involved in the uptake of virulent C. burnetii by macrophages and C. burnetii-stimulated F-actin reorganization. However, TLR4 did not control the microbicidal activity of macrophages toward C. burnetii and, in vivo, tissue infection and bacterial clearance. TLR4 was in contrast necessary for the formation of protective granulomas and the production of IFN-γ and TNF. Hence, TLR4 likely plays a critical role in the early responses of the host to C. burnetii infection by enabling it to develop protective immune responses, but the control of late phases of the infection requires other mechanisms.

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