Link between Impaired Maturation of Phagosomes and Defective Coxiella burnetii Killing in Patients with Chronic Q Fever

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Q fever is caused by Coxiella burnetii, a bacterium that survives in monocytes/macrophages by resisting their natural microbicidal activity. Because the link between bacterial killing and phagosome maturation has yet to be demonstrated, we evaluated responses in monocytes from both immunologically naive control subjects and patients with various manifestations of Q fever. Monocytes from patients with chronic Q fever in evolution, who do not control the infection, exhibited defective phagosome maturation and impaired C. burnetii killing. Both responses were stimulated in patients recovering from Q fever. Phagosome maturation and C. burnetii killing were significantly correlated. Defective phagosome maturation and impaired C. burnetii killing were induced by adding interleukin (IL)–10 to monocytes from convalescent patients and were restored by IL-10 neutralization in chronic Q fever in evolution. We show that phagosome maturation and microbial killing are linked in Q fever and that IL-10 regulates both features of microbicidal activity.

Q fever is a zoonosis caused by Coxiella burnetii, an obligate intracellular bacterium that is considered as a potential biological weapon of group B [1]. The disease is characterized by a primary infection that spontaneously resolves in the vast majority of cases and that may become chronic in a minority of cases. When it is symptomatic, the primary infection may present as isolated fever, hepatitis, or pneumonia [2, 3]. Q fever may become chronic in patients with valve lesions, arterial aneurysm, or prosthesis [3] and principally manifests as an endocarditis. Immunocompromised patients and pregnant women are at greater risk to develop Q fever endocarditis [4]. The majority of individuals with untreated endocarditis will proceed to death [5]; however, appropriate antibiotic treatment dramatically improves the prognosis [6].

The immune system controls the C. burnetii infection, as manifested by granuloma formation [7] and systemic cell-mediated immune responses, including interferon (IFN)–γ production [8, 9]. The efficient immune response accounts for the recovery after the primary infection. It does not lead to C. burnetii eradication [10]; however, relapses occur in the presence of a depressed cell-mediated immunity, such as that with corticosteroid treatment [11] or during pregnancy [12]. During Q fever endocarditis, cell-mediated immunity is defective, with associated lack of granuloma formation and impaired systemic response [8, 13]. The cytokine network is also altered, and interleukin (IL)–10 is overproduced [14, 15].

The mechanisms of C. burnetii replication in monocytes/macrophages are beginning to be understood [16]. For instance, we recently demonstrated that C. burnetii survives in THP-1 monocytes by interfering with phagosome maturation at the late-endosome/lysosome stage (for a review, see [17]). Indeed, C. burnetii–containing vacuoles do not fuse with lysosomes, because they are devoid of cathepsin D; the acquisition of markers of late endosomes and late endosomes–early lysosomes,
such as lysosome-associated membrane protein–1 (Lamp-1), was conserved [18]; however, we have yet to determine whether *C. burnetii* survival depends on phagosome maturation in Q fever. As the microbicidal activity of monocytes/macrophages is regulated by cell-mediated immune processes, it is likely that cytokines produced during Q fever affect phagosome maturation. Indeed, IFN-γ, which is associated with the control of acute Q fever, stimulates phagosome-lysosome (PL) fusion and enables THP-1 monocytes to kill *C. burnetii* [18]. The ability of IFN-γ to stimulate intracellular killing through the modulation of phagosome maturation has been reported for *Listeria monocytogenes* and *Mycobacterium avium* [19–21], but these studies used naïve macrophages and should not be extrapolated to clinical conditions.

We demonstrate in this article that phagosome maturation and *C. burnetii* killing are correlated in Q fever. Both responses are impaired in patients during chronic Q fever in evolution, whereas they are stimulated in patients after recovery from either acute or chronic Q fever.

**SUBJECTS, MATERIALS, AND METHODS**

**Patients.** Patients included in the present study are described in table 1. They consisted of 10 convalescent patients recovering from acute Q fever and 17 patients with Q fever endocarditis (10 with endocarditis in evolution and 7 with cured endocarditis). The diagnosis of Q fever endocarditis was based on modified Duke University criteria [22], including pathological evidence of endocarditis, a positive echocardiogram, a positive blood culture, and high titers of IgG directed against phase I *C. burnetii* (table 1). All these patients had been subjected to microimmunofluorescence; results are presented as titers.

In the present study, the lag time between diagnosis and inclusion did not exceed 2 months in patients with Q fever endocarditis in evolution. Acute Q fever was diagnosed by detection of IgG (titers were between 100 and 1600) specific for phase II *C. burnetii* (table 1). The study included 10 healthy, seronegative control subjects (6 men and 4 women) with a median age of 45 years (range, 32–61 years). Written, informed consent was obtained from each subject, and the present study was approved by the Ethics Committee of the Université de la Méditerranée.

**Cells and bacteria.** Monocytes were isolated from peripheral blood mononuclear cells by glass adherence in RPMI 1640 medium containing 25 mmol/L HEPES, 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). More than 90% of adherent cells were monocytes. Cells were then cultured for 3 days at 37°C in RPMI 1640 medium supplemented with 10% human AB serum (Sigma Aldrich). The culture procedure was a prerequisite for colocalization analysis because the 3-day culture increased monocyte spreading without eliciting their differentiation. All culture media were checked for the absence of endotoxins by *Limulus* amebocyte lysate assay (Cambrex) [23].

Virulent and avirulent *C. burnetii* organisms (Nine Mile strain) were obtained as described elsewhere [24]. In brief, virulent organisms were isolated from infected mice and cultured in L929 cells for 2 passages, whereas avirulent variants were cultured in L929 cells for repeated passages. Infected cells were sonicated, and bacteria were purified on Renografin gradients. Isolated organisms were then aliquoted and stored at −80°C.

**Infection procedure and determination of *C. burnetii* killing.** Monocytes (10⁴ monocytes/mL) were incubated with *C. burnetii* (200 or 25 avirulent bacteria/cell) for 24 h. This procedure allows for similar phagocytosis rates of virulent and avirulent organisms [24]. Monocytes were then washed to remove free bacteria. This was designated as day 0. Infected cells were again cultured for 72 h. In some experiments, 5 ng/mL human recombinant IL-10 or 10 µg/mL neutralizing anti–IL-10 antibodies (R&D Systems) were included in cultures. The viability of intracellular bacteria was assessed by use of the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes), as described elsewhere [18]. In brief, cell preparations were lysed by hypotonic shock, and lysates were centrifuged at 8000 g for 10 min. Pelleted bacteria were collected, and a combination of SYTO 9 and propidium iodide was added to the organisms. Organism fluorescence was observed, and the results were expressed as the percentage of killed bacteria.

**Cytokine determination.** Adherent monocytes (10⁴ monocytes/assay) were stimulated by heat-killed virulent *C. burnetii*

<table>
<thead>
<tr>
<th>Clinical presentation</th>
<th>Age, years</th>
<th>Sex, M:F</th>
<th><em>C. burnetii</em> antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocarditis in evolution</td>
<td>63 (40–84)</td>
<td>6:4</td>
<td>1600 (800–6400) 3200 (800–12,800)</td>
</tr>
<tr>
<td>Cured endocarditis</td>
<td>71 (64–77)</td>
<td>4:3</td>
<td>400 (400–1600) 1600 (1200–3200)</td>
</tr>
<tr>
<td>Recovering from acute Q fever (convalescent)</td>
<td>40 (22–71)</td>
<td>6:4</td>
<td>100 (50–400) 400 (100–1600)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are median (range), unless otherwise noted. The presence of circulating antibodies (IgG) directed against *Coxella burnetii* in phase I and in phase II was assessed by microimmunofluorescence; results are presented as titers.
at a bacterium:cell ratio of 200:1 for 24 h. Supernatants were assayed for the presence of IL-10 by ELISA, as described by the manufacturer (R&D Systems). The limit of detection was 4 pg/mL, and the intra- and interassay coefficients of variation of the ELISA kits ranged between 5% and 12%.

**Colocalization of C. burnetii with cathepsin D.** Bacterial trafficking was studied by immunofluorescence as follows [18]. Monocytes were infected by *C. burnetii* (200 virulent or 25 avirulent bacteria/cell) for 4 h (designated as hour 0). Monocytes were then washed to remove free organisms and were incubated for various times in the presence or absence of neutralizing antibodies or IL-10. After fixation, cells were permeabilized by 0.1% saponin for 30 min. Human antibodies specific for *C. burnetii* were used at a 1:4000 dilution. The antibodies to intracellular markers were rabbit anti–Lamp-1 antibodies (a gift from M. Fukuda, La Jolla Cancer Research Institute, La Jolla, California) and anti–cathepsin D antibodies (a gift from S. Kornfeld, Washington University School of Medicine, St. Louis, Missouri), both used at a 1:1000 dilution. Primary antibodies were added to cell preparations for 30 min. Bacteria were revealed by Texas Red–conjugated F(ab′)2 anti–human IgG antibodies and intracellular markers by fluorescein isothiocyanate–conjugated F(ab′)2 anti–rabbit IgG antibodies (Jackson Immunoresearch Laboratories). Both antibodies were used at a 1:100 dilution. We examined the colocalization of bacteria and intracellular markers with a laser scanning confocal fluorescence microscope (Leica TCS 4D). Optical sections of images were collected at 1-μm intervals and analyzed using Adobe Photoshop (version 5.5). Approximately 25 *C. burnetii*–containing vacuoles were scored per coverslip, and at least 3 distinct experiments were performed per condition. Results were expressed as the percentage of phagosomes expressing cathepsin D.

**Statistical analysis.** Results were expressed as mean ± SE and compared by the nonparametric Mann-Whitney U test. Differences were considered significant at *P* < .05. Cathepsin D distribution and bacterial killing were analyzed with the nonparametric correlation test (Spearman’s).

**RESULTS**

**PL fusion and C. burnetii fate in chronic Q fever.** PL fusion was assessed by measuring the colocalization of cathepsin D with *C. burnetii*. This approach was validated in monocytes from immunologically naive control subjects infected with avirulent and virulent *C. burnetii*. Cathepsin D was detected in the lumen of 76% ± 8% of phagosomes containing avirulent variants of *C. burnetii* at 24 h after infection. Beyond this time point, the percentage of positive phagosomes remained constant (figure 1A and 1B). In contrast, the percentage of phagosomes containing virulent *C. burnetii* that colocalized with cathepsin D did not exceed 22%, whatever the postinfection time (figure 1B). Consequently, all subsequent colocalization studies were performed at 24 h after infection, using virulent organisms. In monocytes from patients with Q fever endocarditis in evolution, the percentage of phagosomes expressing cathepsin D never exceeded 25% (figure 1C). Increasing the culture time to 72 h had no effect on the percentage of positive phagosomes (data not shown). This percentage was similar to that of monocytes from naive control subjects. It was markedly distinct from monocytes isolated from convalescent patients recovering from acute Q fever, in which efficient PL fusion was observed (figure 1C). Hence, in convalescent patients, the percentage of phagosomes expressing cathepsin D was initially high, and >50% of phagosomes had acquired cathepsin D after 24 h. The percentage was significantly higher than that in control subjects (*P* < .004) and patients with chronic Q fever in evolution (*P* < .0001). It is worthwhile to note that the interaction of phagosomes with Lamp-1 was not affected: the percentage of phagosomes expressing Lamp-1 reached 100% after 24 h in control subjects, patients recovering from Q fever, and patients with Q fever endocarditis in evolution.

As described elsewhere [24], we found that monocytes from immunologically naive control subjects killed avirulent *C. burnetii* organisms but were unable to kill virulent organisms (figure 1B). In patients with Q fever endocarditis in evolution, the percentage of virulent organisms that were killed by monocytes did not exceed 25%. It was similar to that found for monocytes from naive control subjects (figure 1C). In contrast, the killing of virulent *C. burnetii* reached 50% in monocytes from convalescent patients. It was significantly higher (*P* < .002) than that in patients with chronic Q fever in evolution.

The percentage of phagosomes expressing cathepsin D was significantly higher (*P* < .0001) in patients who recovered from Q fever endocarditis than in patients with Q fever endocarditis in evolution, and it reached that in convalescent patients recovering from acute Q fever (figure 1C). However, their microbicidal competence was only partly restored. Hence, the percentage of virulent organisms that were killed was significantly higher (*P* < .05) in cured patients than in patients with endocarditis in evolution and was significantly lower (*P* < .004) than in convalescent patients recovering from acute Q fever. When all the results of cathepsin D distribution and *C. burnetii* killing from control subjects and patients were plotted (figure 1D), there was a clear correlation between both events (*r* = 0.694; *P* < .0001). Taken together, these results show that *C. burnetii* killing by monocytes is associated with PL fusion.

**Dependence of PL fusion and C. burnetii killing on IL-10.** Because IL-10 is likely involved in the microbicidal defect of monocytes observed in Q fever endocarditis [16], its potential role in defective PL fusion was studied. First, the unstimulated production of IL-10 by monocytes was markedly higher than that by monocytes from naive control subjects (*P* < .04) and convalescent patients recovering from acute Q fever (*P* < .002).
Figure 1. Phagosome-lysosome fusion and Coxiella burnetii fate in Q fever. A and B, Monocytes from immunologically naive control subjects were incubated with C. burnetii, washed, and cultured for various times. A, Avirulent bacteria (top panel) and cathepsin D (middle panel) revealed by indirect immunofluorescence and confocal microscopic analysis. The colocalization of cathepsin D with bacteria was confirmed by merging images (bottom panel). The inset shows the distribution of cathepsin D. B, Results expressed as the percentage of C. burnetii phagosomes expressing cathepsin D (top panel). The microbicidal activity of monocytes was determined by use of a bacterial viability kit, and the results are expressed as the percentage of killed bacteria (bottom panel). The results represent the mean ± SE of 5 experiments. C, Monocytes from naive control subjects, convalescent patients recovering from acute Q fever, and patients with Q fever endocarditis in evolution or cured Q fever endocarditis incubated with virulent C. burnetii, washed, and cultured for various times. The colocalization of C. burnetii with cathepsin D (top panel) and the microbicidal activity of monocytes toward C. burnetii (bottom panel) were determined as described above. D, Positive correlation between the percentage of phagosomes expressing cathepsin D and C. burnetii killing for each studied individual (r = 0.694; P < .0001).

Similarly, the release of IL-10 by monocytes stimulated with heat-killed virulent C. burnetii was significantly higher in patients with Q fever endocarditis in evolution than in naive control subjects (P < .03) or convalescent patients recovering from acute Q fever (P < .004) (figure 2A). Second, IL-10 neutralization with anti–IL-10 antibodies restored PL fusion and C. burnetii killing in monocytes from patients with Q fever endocarditis in evolution. Indeed, in the presence of anti–IL-10 antibodies, the percentage of phagosomes expressing cathepsin D was increased 3.2-fold (P < .0002; figure 2B). The same treatment was less efficient in monocytes from convalescent patients recovering from acute Q fever (1.4-fold increase; P < .002) and in monocytes from naive control subjects (2.5-fold increase; P < .01). The neutralization of endogenous IL-10 also affected the killing of virulent C. burnetii, which was dramatically increased (3-fold; P < .0003) in patients with Q fever endocarditis in evolution (figure 2B). It moderately increased bacterial killing in monocytes from naive control subjects and convalescent patients (P < .01). The treatment of monocytes with anti–tumor necrosis factor antibodies, used as negative controls, had no effect on PL fusion or C. burnetii killing (data not shown). Third, the addition of IL-10 to monocytes from convalescent patients recovering from acute Q fever inhibited both PL fusion and C. burnetii killing. Hence, the percentage of phagosomes expressing cathepsin D was decreased by 54% (P < .003) and bacterial killing was decreased by 59% (P < .003; figure 2C). In contrast, the addition of IL-10 to monocytes from naive control subjects and patients with Q fever endocarditis in evolution had no effect. These results show that defective PL fusion and impaired C. burnetii killing in Q fever...
endocarditis were both caused by IL-10 and were restored by IL-10 neutralization.

**DISCUSSION**

The ability of macrophages to kill invading microorganisms has been related to the maturation of phagosomes toward phagolysosomes. Several intracellular pathogens that escape the microbicidal activity of macrophages are known to prevent PL fusion [17]. Nevertheless, this concept is based on results obtained with immunologically naive monocytes/macrophages, and the link between phagosome maturation and macrophage microbicidal activity is lacking in vivo. We have shown here that phagosome maturation and microbial killing of macrophages were correlated in Q fever, a paradigm of chronic infectious diseases. Indeed, monocytes from convalescent patients recovering from acute Q fever exhibited fusion of *C. burnetii* phagosomes with lysosomes and, subsequently, efficient bacterial killing. This finding highlights previously reported *C. burnetii* clearance in patients with acute Q fever [3]. In contrast, monocytes from patients with chronic Q fever in evolution had defective PL fusion and impaired *C. burnetii* killing. This finding extends our previous reports that showed impaired microbial killing in patients with chronic Q fever in evolution [15]. The microbicidal defect is not constitutive but is related to the activity of the disease. Indeed, in patients who recovered from Q fever endocarditis, defective PL fusion and impaired *C. burnetii* killing were restored.

We have provided evidence that phagosome maturation and *C. burnetii* killing were modulated by IL-10. Indeed, IL-10 was overproduced in patients with Q fever endocarditis in evolution. Neutralization of endogenous IL-10 restored PL fusion and enhanced *C. burnetii* killing by monocytes, which thus behaved as did those from convalescent patients. This is consistent with the fact that IL-10 directly affects PL fusion and *C. burnetii* killing. Indeed, the addition of IL-10 to monocytes from convalescent patients recovering from acute Q fever prevented the induction of phagosome maturation and *C. burnetii* killing. Previous reports have described several effects of IL-10 on endocytosis and intracellular traffic. IL-10 affects fluid-phase and mannose receptor–mediated endocytosis and decreases particle sorting to perinuclear lysosomes in human primary macrophages [25]. IL-10 is involved in the re-endocytosis and the intracellular retention of HLA-DR molecules by monocytes during septic shock [26]. IL-10 decreases the expression of precursor and mature-form cathepsin D in peripheral monocytes and lamina propria mononuclear cells from patients with...
inflammatory bowel disease [27]. More specifically, the maturation of phagosomes containing mycobacteria is improved in macrophages from IL-10–deficient mice [21].

Our results demonstrate, for the first time, a direct link between phagosome maturation and microbial competence in vivo. They also show that phagosome maturation reflects the efficiency of the immune response. During chronic Q fever, in which the infection is not controlled, both C. burnetii trafficking and killing are defective. The deficiency is caused by IL-10 overproduction and is restored by IL-10 neutralization, thus demonstrating the role of cytokines in phagosome maturation.

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References