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.
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 micbicidal activity of monocytes/macrophages
is regulated by cell-mediated immune processes, it is likely that
cytokines produced during Q fever affect phagosome matu-
ration. 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 modu-
lation of phagosome maturation has been reported for Listeria
monocytogenes and Mycobacterium avium [19–21], but these
studies used naive 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 endocar-
ditis). The diagnosis of Q fever endocarditis was based on mod-
ified Duke University criteria [22], including pathological evi-
dence 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
valve replacement and medical treatment consisting of doxy-
cycline and hydroxychloroquine because this regimen shortens
the duration of the treatment and reduces the number of re-
lapses [6]. 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 de-
tection of IgG (titers were between 100 and 500) 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 periph-
eral blood mononuclear cells by glass adherence in RPMI 1640
medium containing 25 mmol/L HEPES, 10% fetal bovine se-
rum, 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 prereq-
quisite for colocalization analysis because the 3-day culture in-
creased monocyte spreading without eliciting their differen-
tiation. 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 cul-
tured 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 gra-
dients. 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. bur-
etii (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 then
cultured for 72 h. In some experiments, 5 ng/mL human re-
combinant IL-10 or 10 μg/mL neutralizing anti–IL-10 anti-
odies (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 hy-
potonic 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 ex-
pressed as the percentage of killed bacteria.

Cytokine determination. Adherent monocytes (10⁴ mon-
ocytes/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>C. burnetii antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocarditis in evolution</td>
<td>63 (40–84)</td>
<td>6:4</td>
<td>1600 (800–6400)</td>
</tr>
<tr>
<td>Cured endocarditis</td>
<td>71 (64–77)</td>
<td>4:3</td>
<td>400 (400–1600)</td>
</tr>
<tr>
<td>Recovering from acute Q fever (convalescent)</td>
<td>40 (22–71)</td>
<td>6:4</td>
<td>100 (50–400)</td>
</tr>
</tbody>
</table>

NOTE. Data are median (range), unless otherwise noted. The presence of circulating antibodies (IgG) directed against Coxiella
burnetii in phase I and in phase II was assessed by micromunofluorescence; 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 < .04) 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 < .01), 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 **Figure 2.** Role of interleukin (IL–10) in phagosome-lysosome fusion and *Coxiella burnetii* fate. A, Monocytes from immunologically naive control subjects, convalescent patients recovering from acute Q fever, and patients with Q fever endocarditis in evolution incubated with or without heat-killed virulent *C. burnetii*. IL-10 was measured in supernatants by immunoassay. B, Monocytes from naive control subjects, convalescent patients recovering from acute Q fever, and patients with Q fever endocarditis in evolution incubated with virulent *C. burnetii*, washed, and cultured in the presence or absence of neutralizing anti–IL-10 antibodies. 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 in figure 1. C, Monocytes from convalescent patients recovering from acute Q fever incubated with virulent *C. burnetii*, washed, and cultured in the presence or absence of recombinant IL-10. The colocalization of *C. burnetii* with cathepsin D (top panel) and the microbicidal activity of monocytes toward *C. burnetii* (bottom panel) were determined.
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**