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Coxiella burnetii Survival in THP-1 Monocytes Involves the Impairment of Phagosome Maturation: IFN-γ Mediates its Restoration and Bacterial Killing[1]

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The subversion of microbicidal functions of macrophages by intracellular pathogens is critical for their survival and pathogenicity. The replication of *Coxiella burnetii*, the agent of Q fever, in acidic phagolysosomes of nonphagocytic cells has been considered as a paradigm of intracellular life of bacteria. We show in this study that *C. burnetii* survival in THP-1 monocytes was not related to phagosomal pH because bacterial vacuoles were acidic independently of *C. burnetii* virulence. In contrast, virulent *C. burnetii* escapes killing in resting THP-1 cells by preventing phagosome maturation. Indeed, *C. burnetii* vacuoles did not fuse with lysosomes because they were devoid of cathepsin D, and did not accumulate lysosomal trackers; the acquisition of markers of late endosomes and late endosomes-early lysosomes was conserved. In contrast, avirulent variants of *C. burnetii* were eliminated by monocytes and their vacuoles accumulated late endosomal and lysosomal markers. The fate of virulent *C. burnetii* in THP-1 monocytes depends on cell activation. Monocyte activation by IFN-γ restored *C. burnetii* killing and phagosome maturation as assessed by colocalization of *C. burnetii* with active cathepsin D. In addition, when IFN-γ was added before cell infection, it was able to stimulate *C. burnetii* killing but it also induced vacuolar alkalinization. These findings suggest that IFN-γ mediates *C. burnetii* killing via two distinct mechanisms, phagosome maturation, and phagosome alkalinization. Thus, the tuning of vacuole biogenesis is likely a key part of *C. burnetii* survival and the pathophysiology of Q fever. *The Journal of Immunology*, 2002, 169: 4488–4495.

Coxiella burnetii, an obligate intracellular Gram-negative bacterium classified in the γ subdivision of proteobacteria, is the agent of Q fever (1). Whereas acute Q fever is controlled by cell-mediated immunity, this latter is defective in chronic Q fever (2). The establishment of *C. burnetii* infection is based on a specific strategy of invasion of monocytes/macrophages. Virulent organisms are poorly internalized by macrophages and their uptake requires the engagement of $\alpha_v\beta_3$ integrin; avirulent variants are efficiently internalized through $\alpha_v\beta_3$ integrin and complement receptor type 3. The selective use of phagocytic receptors is an active process based on interference with complement receptor type 3-mediated phagocytosis (3). Once internalized, *C. burnetii* survives and replicates in an acidic environment (4, 5), which is needed for bacterial metabolism (6, 7). This low pH also accounts for the relative inefficiency of antibiotics toward *C. burnetii* (1). The association of acidic pH and phagolysosomal features has led most authors to consider the intracellular life of *C. burnetii* as a paradigm of intracellular survival without alteration of intracellular traffic (5). However, most of these studies were performed with avirulent *C. burnetii* (8, 9). In addition, these studies used fibroblasts and murine macrophage-like cells, in which virulent and avirulent *C. burnetii* replicate (5), whereas only virulent *C. burnetii* survives in human monocytes/macrophages (3).

The fate of intracellular microorganisms including *C. burnetii* depends on the microbicidal properties of macrophages and their regulation by cytokines. A defective killing of *C. burnetii* was found in monocytes from patients with chronic Q fever (10), which partly results from IL-10-mediated impairment of macrophage microbicidal activity. Indeed, IL-10 elicits the replication of *C. burnetii* in resting monocytes, and neutralizing anti-IL-10 Abs restore microbicidal activity against *C. burnetii* in patients with chronic Q fever (11). In contrast, IFN-γ, known to stimulate the microbicidal activity of macrophages, triggers *C. burnetii* killing in THP-1 monocytes (12). The ability of IFN-γ to stimulate the microbicidal activity of macrophages has been related to oxygen-dependent mechanisms (13), but reactive oxygen intermediates are not involved in the killing of *C. burnetii* (12). As IFN-γ-induced killing of *Listeria monocytogenes* and *Mycobacterium avium* has been associated with the modulation of phagosome maturation (14–16), we hypothesized that such mechanisms may be involved in *C. burnetii* killing.

We show in this study that the survival of *C. burnetii* in THP-1 monocytes is associated with altered phagosome maturation. *C. burnetii* organisms are present in phagosomes that acquire markers of late endosomes and late endosomes-early lysosomes but not the lysosomal enzyme cathepsin D. The survival of *C. burnetii* depends on the activation of THP-1 cells. Indeed, IFN-γ induces *C. burnetii* killing and restores phagolysosomal fusion. This study also provides evidence that IFN-γ-induced killing of *C. burnetii* involves two distinct mechanisms, phagosome maturation and late phagosome alkalinization.

Materials and Methods

Cells and bacteria

THP-1 monocytic cells were cultured as previously described (12). Cells (5×10^4 cells/assay) were seeded on 12-mm round coverslips in flat-bottom 24-well plates (Nunc, Roskilde, Denmark) and were treated with 10

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ng/ml PMA (Sigma-Aldrich, St. Louis, MO) to become adherent. After 24 h at 37°C, cells were washed three times in antibiotic-free RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine (Invitrogen, Eragny France). PBMC were isolated from healthy volunteers on Ficoll gradient (MSL, Eurobio, Les Ullis, France), and monocytes were purified by adherence on glass Labtek chamber/slides (Miles, Naperville IL), as previously described (11). Nonadherent cells were removed by washing, and remaining cells were cultured for 3 days in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine. Virulent and avirulent *C. burnetii* organisms (Nine Mile strain, ATCC VR-615; American Type Culture Collection, Manassas, VA) were obtained as previously described (3). In brief, virulent organisms were isolated from infected mice and cultured in L929 cells for two passages whereas avirulent variants were cultured in L929 cells by repeated passages. Two other virulent strains of *C. burnetii*, Priscilla and Q212, isolated from an infected goat and a patient with acute Q fever, respectively, were cultured like the virulent Nine Mile strain. Bacteria were layered on 25–45% linear Renograffin gradient, and the gradients were spun down. Purified bacteria were then collected, washed, and suspended in HBSS before being stored at −80°C. The number of bacteria was determined by Gimenez staining. The viability of *C. burnetii* was assessed using the LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes, Eugene, OR) as recommended by the manufacturer. Briefly, the *C. burnetii* suspension was incubated with SYTO 9 stain and propidium iodide, and examined with a fluorescence microscope. Results are expressed as the ratio of viable bacteria and the total number of bacteria. Only *C. burnetii* preparations containing >90% of viable organisms were used. Heat-killed virulent organisms were obtained by heating the bacterial suspension at 100°C for 1 h, and were stored at −80°C.

Intracellular fate of C. burnetii

THP-1 cells and monocytes from healthy donors were incubated with *C. burnetii* in antibiotic-free RPMI 1640 containing 10% FCS. After 24 h at 37°C, cells were washed to remove free bacteria (this time was designated as day 0): this procedure was sufficient to remove noninternalized and loosely attached organisms (3). Monocytic cells were again cultured for different periods. In some experiments, THP-1 cells were incubated with human rIFN-γ (R&D Systems, Abingdon, U.K.) for 16 h, and then infected with *C. burnetii*. After 24 h, cells were washed to remove free bacteria (corresponding to day 0), and were again incubated with IFN-γ. Alternatively, IFN-γ was added to infected cells and the same procedure was used. Intracellular bacteria were revealed by indirect immunofluorescence. Briefly, cell preparations were fixed with 1% formaldehyde, incubated with human Abs to *C. burnetii* (purified IgG from patients with Q fever endocarditis, 1/4000 dilution) in the presence or the absence of 0.1 mg/ml lysophosphatidylcholine, washed, and incubated with a 1/200 dilution of FITC-conjugated $F(ab')_2$ anti-human IgG Abs (Beckman Coulter, Roissy, France). Results are expressed as an infection index, which is the product of the mean number of bacteria per infected cell and the percentage of infected cells × 100 (3). The viability of intracellular bacteria was assessed using the bacterial viability kit. The infected cells were homogenized in water and vigorously mixed. The cell lysate was centrifuged at 8000 × *g* for 10 min, and pelleted bacteria were collected. The combination of SYTO 9 stain and propidium iodide was added to the bacterial suspension and the fluorescence of organisms was observed. Results are expressed in percentage of live bacteria.

Phagosome acidification

The phagosome acidification was analyzed using DM-NERF dextran (molecular mass, 10 kDa; Molecular Probes), a fluorescent probe of phagosomal pH (17). THP-1 cells were incubated with 20 μg/ml DM-NERF dextran and *C. burnetii*, or latex beads (0.8 μm; Sigma-Aldrich) as control, for 24 h. In some experiments, infected cells loaded with DM-NERF dextran were incubated with 10 nM bafilomycin A1 (Sigma-Aldrich), a specific inhibitor of vacuolar proton ATPase ($V\text{-}H^+$-ATPase)[3] (18) for 2 h. Bacteria were revealed by human Abs to *C. burnetii* and Texas Red-conjugated $F(ab')_2$ anti-human IgG Abs (Beckman Coulter) used at a 1/100 dilution. The intraphagosomal pH was measured by ratiometric analysis of fluorescence intensities of DM-NERF dextran (excitation, 490/440 nm; emission, 530 nm). Infected cells were incubated with buffer solutions with graded pH (4.0, 5.0, 6.0, 7.0, and 7.4) in the absence or the presence of 10 μM monensin (Sigma-Aldrich), which equilibrated the intravacuolar pH with extracellular pH (19). After 1 h, fluorescence was recovered using a microplate fluorescent reader (Fisher Scientific, Elancourt, France). The mean pH value of the samples was calculated using a reference pH curve.

Bacterial trafficking

Bacterial trafficking was studied by immunofluorescence as follows (20). THP-1 cells and monocytes were infected by *C. burnetii* (200 virulent bacteria or 25 avirulent bacteria per cell) for 4 h (considered as h 0), washed to remove free organisms, and incubated for additional periods. Cell preparations were then fixed in 3% paraformaldehyde for 20 min. After washing, cells were incubated with ammonium chloride to neutralize free aldehydes and were permeabilized by PBS containing 0.1% saponin and 10% horse serum for 30 min. Human Abs specific for *C. burnetii* were used at a 1/4000 dilution. The Abs to intracellular markers were: rabbit anti-lysosome-associated membrane protein-1 (Lamp-1) Abs (a gift from Dr. M. Fukuda, The Burnham Institute, La Jolla, CA) used at a 1/1000 dilution, anti-cation-independent mannose-6-phosphate R (M6PR) Abs (a gift from Dr. B. Hoflack, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) used at a 1/500 dilution, anti-Rab7 Abs (a gift from Dr. M. Zerial, Max Planck Institute of Molecular Cell Biology and Genetics) used at a 1/200 dilution, anti-cathepsin D Abs (a gift from Dr. S. Kornfeld, Washington University School of Medicine, St. Louis, MO) used at a 1/1000 dilution; mouse anti-CD63 mAbs (BD Biosciences, le Pont de Claix, France) used at a 1/1000 dilution, and anti-$V\text{-}H^+$-ATPase mAbs (Chemicon International, Temecula, CA) used at a 1:500 dilution. Primary Abs were added to cell preparations in PBS containing 0.1% saponin and 5% horse serum for 30 min. After being washed, monocytic cells were incubated with fluorescent secondary Abs in 0.1% saponin. Bacteria were revealed by Texas Red-conjugated $F(ab')_2$ anti-human IgG Abs and intracellular markers by FITC-conjugated $F(ab')_2$ anti-rabbit or anti-mouse IgG Abs (Beckman Coulter), both Abs being used at a 1:100 dilution. The colocalization of bacteria with intracellular markers was examined with a laser scanning confocal fluorescence microscope (Leica TCS 4D; Heidelberg, Germany). Optical sections of images were collected at 0.5-μm intervals and analyzed using Adobe Photoshop V5.5 software (Mountain View, CA). *C. burnetii* phagosomes were scored as positive for soluble markers when fluorescence was observed in the phagosome lumen; for membrane markers, phagosomes were scored as positive when a fluorescence ring surrounded organisms. About 30 *C. burnetii*-containing vacuoles were scored per coverslip, and at least three distinct experiments were performed per condition. Results are expressed as the percentage of phagosomes expressing intracellular markers.

Lysosomal tracker and in situ measurement of cathepsin D activity

THP-1 cells were infected with *C. burnetii* for 4 h. The lysosomotropic probe neutral red (Molecular Probes) was added at 5 μg/ml to infected cells 2 h before the end of the infection time. After being washed, cells were fixed in 3% paraformaldehyde. Bacteria were revealed by indirect immunofluorescence with FITC-conjugated secondary Abs, and neutral red was observed with excitation and emission filters for Texas Red. The cathepsin D-sensitive near-infrared fluorescence (NIRF) probe was prepared as previously described (21, 22). It was conjugated with FITC to monitor probe internalization and with a Cy5.5 marker that became fluorescent in the near-infrared spectrum after cathepsin D activation. NIRF at 0.1 μM was added to cells 1 h before the end of the infection time. THP-1 cells were then washed to remove noninternalized organisms and the free NIRF probe and were fixed with methanol at −20°C for 5 min. Bacteria were revealed by indirect immunofluorescence with Texas Red-conjugated secondary Abs. The colocalization of bacteria with the NIRF probe was examined with the laser scanning confocal fluorescence microscope equipped with appropriate excitation and emission filters for FITC, Texas Red, and Cy5.5. Images were analyzed using Adobe Photoshop version 5.5 software.

Statistical analysis

Results, given as mean ± SE, were compared with Student's *t* test. Differences were considered significant when $p < 0.05$.

Results

Virulent C. burnetii, *but not avirulent variants, survives in adherent THP-1 cells*

As suspended THP-1 cells are not suitable tools for studying intracellular traffic of *C. burnetii*, they were treated by PMA to induce their spreading and adhesion, and then incubated with *C. burnetii*. Adding virulent organisms (200:1 bacterium-cell ratio) to adherent THP-1 cells for 24 h led to the infection of 85 ± 6% of

[3] Abbreviations used in this paper: $V\text{-}H^+$-ATPase, vacuolar proton ATPase; Lamp, lysosome-associated membrane protein; M6PR, cation-independent mannose-6-phosphate R; NIRF, near-infrared fluorescence.

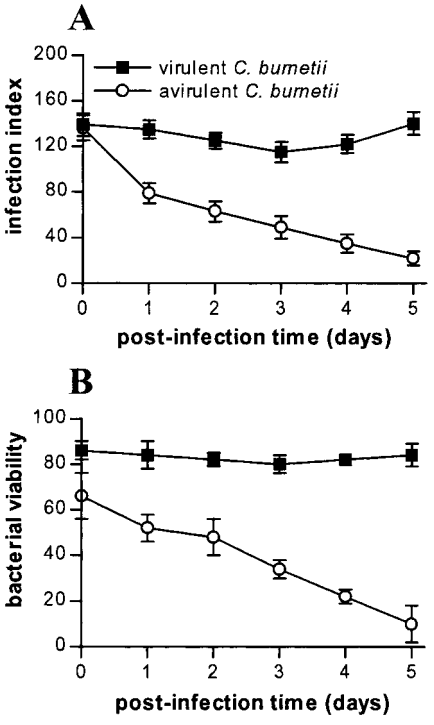

FIGURE 1. *C. burnetii* survival in THP-1 cells. THP-1 cells were incubated with virulent or avirulent *C. burnetii* for 24 h (designated as day 0), and then cultured for 5 days. *A*, Intracellular bacteria were revealed by immunofluorescence. The infection index was determined as the product of the mean number of bacteria per infected cell and the percentage of infected cells × 100. The results are the mean ± SE of four experiments. *B*, Infected cells were homogenized, and viable and dead bacteria were revealed by SYTO9 and propidium iodide, respectively. Results are expressed in percentage of viable bacteria. They are the mean ± SE of four experiments.

cells with 1–2 bacteria per cell. Cellular infection slightly decreased from days 0 to 3 by 17% and then steadily reached the initial value of infection after 5 days (Fig. 1*A*). Beyond this time, a decrease in viability of THP-1 cells impaired the determination of cellular infection. The changes in bacterial number correlated with bacterial viability, which was assessed under the same experimental conditions. The percentage of live virulent organisms did not vary over 5 days (Fig. 1*B*). In contrast, avirulent variants of *C. burnetii* were eliminated by adherent THP-1 cells. As they were more efficiently internalized than virulent organisms, they were added to THP-1 cells at a bacterium-cell ratio of 25:1. At day 0, 84 ± 8% of cells were infected with 1 or 2 bacteria, and cellular infection decreased by 42% at day 1 and slowly went down to reach 84% inhibition at day 5 (Fig. 1*A*). The viability of avirulent organisms rapidly decreased during the course of experiments because it was diminished by 35% at day 0 and 84% at day 5 (Fig. 1*B*). These results indicate that only virulent *C. burnetii* survived in PMA-treated THP-1 cells.

C. burnetii *organisms are present in acidic vacuoles*

As previous reports suggested that *C. burnetii* replicates in acidic compartments (5), we investigated the relationship between acidic pH and *C. burnetii* survival in adherent THP-1 cells. The intravacuolar pH was determined by ratiometric analysis of fluorescence intensities of pH-sensitive DM-NERF dextran. In uninfected THP-1 cells, the pH of phagosomes containing latex beads was acidic. After 24 h of incubation of THP-1 cells with *C. burnetii*, intravacuolar pH was 5.0 ± 0.1 for virulent organisms and 5.2 ± 0.1 for avirulent organisms (Table I). The acidic pH of *C. burnetii* vacuoles results from the acquisition of V-H^+-ATPase, known to acidify phagosomes. The percentage of vacuoles that accumulated V-H^+-ATPase was 58 ± 11% at h 0, and it progressively increased to 80 ± 8% at h 72. V-H^+-ATPase also colocalized with avirulent *C. burnetii*: 72 ± 10 and 88 ± 8% of vacuoles containing avirulent organisms colocalized with V-H^+-ATPase at hours 0 and 72, respectively (Fig. 2). The V-H^+-ATPase was functional as demonstrated by using bafilomycin A1, a specific inhibitor of V-H^+-ATPase. In the presence of bafilomycin A1, the pH of vacuoles containing virulent or avirulent *C. burnetii* was significantly ($p < 0.02$) higher than that in the absence of bafilomycin A1 (Table I). Hence, *C. burnetii* is present in vacuoles that are acidified by V-H^+-ATPase, independently of organism virulence.

Virulent C. burnetii *is present in nonlysosomal vacuoles*

As the acidic pH of *C. burnetii* vacuoles cannot account for the survival of virulent organisms in THP-1 cells, we suggested that the dynamics of vacuoles containing virulent organisms is distinct from that of avirulent organisms. The intracellular traffic of *C. burnetii* vacuoles was studied by measurement of organism colocalization with the lysosomal protease cathepsin D. Cathepsin D did not accumulate in vacuoles containing virulent *C. burnetii* (Fig. 3*A*). At h 0, only 10 ± 5% of virulent *C. burnetii* colocalized with cathepsin D, but cathepsin D appeared in the lumen of 42 ± 6% of vacuoles containing avirulent organisms (Fig. 3*A*). It is noteworthy that the amount of infection with avirulent *C. burnetii* remained higher than in cells infected with virulent organisms as a consequence of distinct phagocytosis efficiency. The lack of cathepsin D colocalization with *C. burnetii* was not due to its delayed acquisition. Indeed, the percentage of vacuoles containing virulent *C. burnetii* that colocalized with cathepsin D did not exceed 20% whatever the postinfection time, but it steadily increased in cells infected with avirulent variants, reaching 88 ± 7% after 96 h (Fig. 3*B*). To confirm that defective acquisition of cathepsin D corresponds to impaired phagosome-lysosome fusion, two probes that accumulated in the lysosomal compartment were used. First, whereas the

Table I. *pH of* C. burnetii *phagosomes*[a]

	− IFN-γ		+ IFN-γ	
	− baf A1	+ baf A1	Before infection	After infection
Latex beads	4.4 ± 0.4	6.5 ± 0.1*	4.9 ± 0.6	4.5 ± 0.3
Virulent *C. burnetii*	5.0 ± 0.1	6.3 ± 0.1*	6.2 ± 0.3	4.8 ± 0.4
Avirulent *C. burnetii*	5.2 ± 0.1	6.6 ± 0.1*	6.2 ± 0.3	nd

[a] THP-1 cells, treated or not by IFN-γ (200 U/ml), were incubated with *C. burnetii* or latex beads in the presence of DM-NERF dextran for 24 h. The results are expressed as pH units and are the means ± SE of three experiments.
*, $p < 0.05$ represents the comparison of pH values in the presence and the absence of bafilomycin A1 (baf A1). nd, not done.

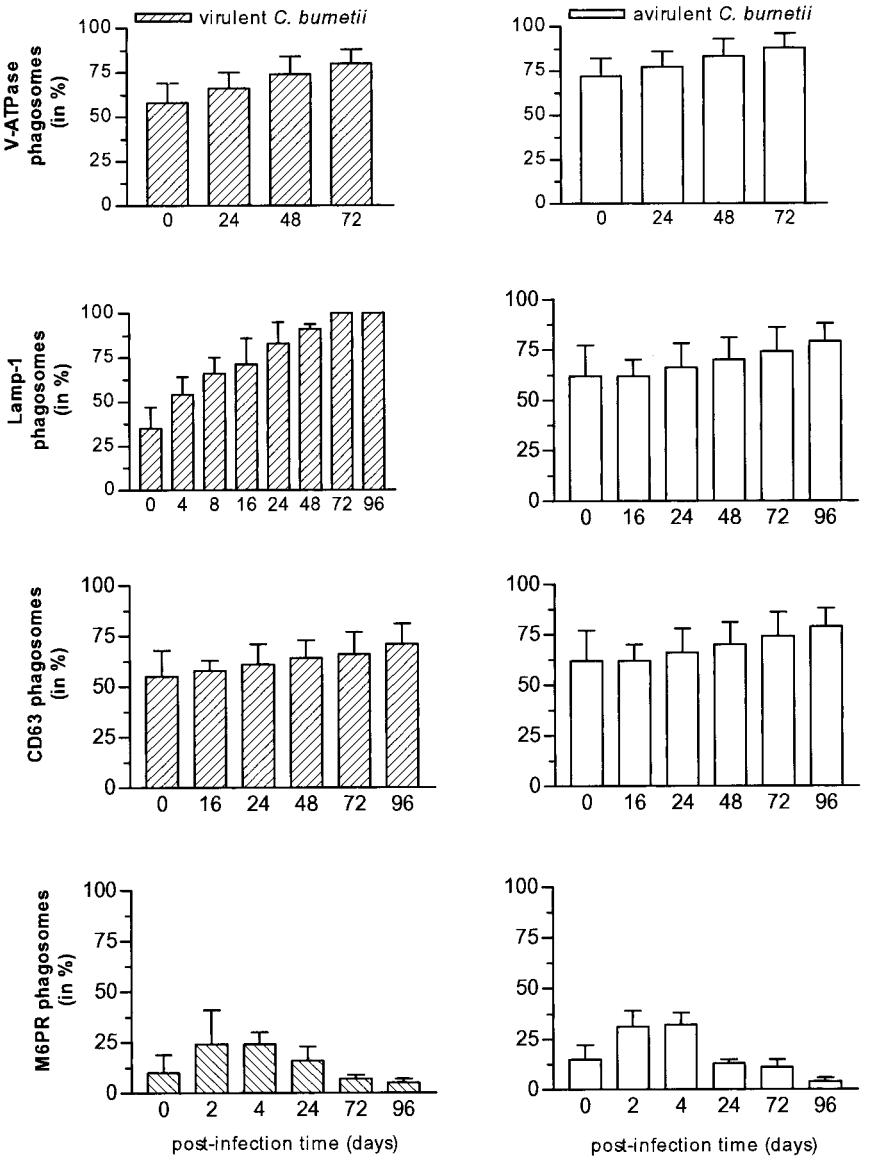

FIGURE 2. Colocalization of *C. burnetii* with late endosome markers. THP-1 cells were infected by virulent and avirulent *C. burnetii* for 4 h, washed to remove free bacteria, and cultured 72 or 96 h. Bacteria and marker proteins were revealed by indirect immunofluorescence, and their colocalization was analyzed by confocal microscopy. Results are expressed as the percentage of phagosomes positive for the marker. They are the mean ± SE of three experiments.

NIRF probe did not colocalize with virulent organisms, it accumulated in phagosomes containing avirulent organisms (Fig. 4*A*). Second, the lysosomotropic probe neutral red colocalized only with phagosomes containing avirulent variants of *C. burnetii* (Fig. 4*B*). Defective phagosome-lysosome fusion was not strain-dependent. Indeed, the percentage of phagosomes containing organisms from Priscilla and Q212 strains that colocalized with cathepsin D was 10 ± 3% and 15 ± 4%, respectively (data not shown). In addition, when virulent *C. burnetii* organisms were heat-killed, they regained the ability to colocalize with cathepsin D (33 ± 5% of positive vacuoles at day 0 and 80 ± 9% of positive vacuoles after 96 h). Hence, defective phagosome-lysosome fusion was related to *C. burnetii* virulence.

To determine whether impaired access of *C. burnetii* vacuoles to late endosomes accounts for defective acquisition of cathepsin D, we studied *C. burnetii* colocalization with markers of late endosomes-early lysosomes, Lamp-1 and CD63. The Lamp-1 fluorescence appeared as a ring surrounding the organisms, and 35 ± 12% of phagosomes containing virulent *C. burnetii* colocalized with Lamp-1 at h 0. The percentage progressively increased, and all bacteria were colocalized with Lamp-1 at h 72 (Fig. 2). The colocalization of avirulent *C. burnetii* with Lamp-1 was similar to that of virulent organisms. In addition, *C. burnetii* colocalized with CD63: the colocalization was high at h 0 (55 ± 13% for virulent organisms and 62 ± 15% for avirulent organisms), and it remained elevated during the incubation time (Fig. 2). M6PR is a marker of late endosomes that is transiently acquired by phagosomes containing inert particles. After 2 h, 24 ± 17% of phagosomes containing virulent *C. burnetii* and 27 ± 10% of phagosomes containing avirulent variants expressed M6PR. Although the percentage of vacuoles containing *C. burnetii* organisms that expressed M6PR remained low, it was similar in cells infected with virulent or avirulent organisms (Fig. 2). Hence, the acquisition of Lamp-1, CD63, and M6PR by *C. burnetii* phagosomes was not related to bacterial virulence. Distinct results were obtained with Rab7, a small GTPase involved in phagosome maturation (23). Rab7 was diffusively stained within the cytoplasm and its fluorescence was concentrated around bacteria (Fig. 5*A*). Only 26 ± 9% of vacuoles containing virulent organisms colocalized with Rab7 at h 0; the percentage remained constant until 96 h despite a moderate increase (38 ± 5%) at h 2. In contrast, 65 ± 7% of vacuoles containing avirulent organisms were colocalized with Rab7 at h 0, and this percentage steadily decreased to values similar to those of virulent organisms after 96 h (Fig. 5*B*). Hence, vacuoles containing virulent *C. burnetii* partially acquired Rab7.

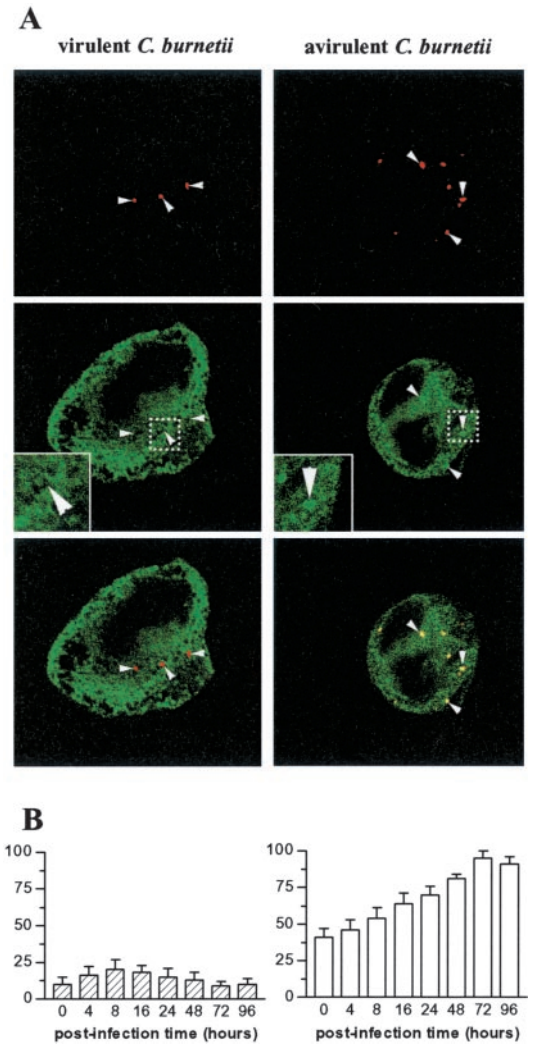

FIGURE 3. Colocalization of *C. burnetii* with cathepsin D. THP-1 cells were infected by virulent and avirulent *C. burnetii* for 4 h. *A*, Bacteria (*top panels*) and cathepsin D (*middle panels*) were revealed by indirect immunofluorescence and examined by confocal microscopy. The colocalization of cathepsin D with bacteria was confirmed by merging fluorescent images (*bottom panels*). Arrows indicate bacteria (*top panels*) and colocalization with cathepsin D (*middle* and *bottom panels*). Insets show the distribution of cathepsin D. *B*, Infected cells were incubated again for 96 h. Results are expressed as the percentage of *C. burnetii* vacuoles expressing cathepsin D. They are the mean ± SE of three experiments.

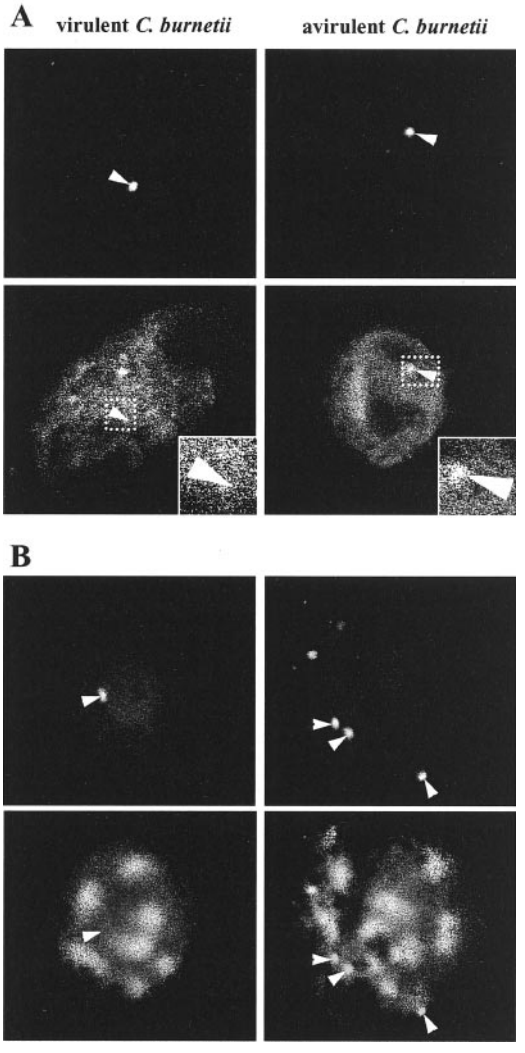

FIGURE 4. Distribution of lysosomal trackers in *C. burnetii*-infected THP-1 cells. THP-1 cells were infected by virulent and avirulent *C. burnetii* organisms for 4 h. NIRF probe (*A*) and neutral red (*B*) were added to cells for 1 and 2 h, respectively. The colocalization of bacteria (*top panels*) with NIRF probe or neutral red (*bottom panels*) was examined with a confocal microscope equipped with appropriate excitation and emission filters for FITC and Texas Red. Arrows indicate bacteria (*top panels*) and colocalization with markers (*bottom panels*). Insets show the distribution of NIRF in a phagosome containing virulent or avirulent *C. burnetii*. The figure is representative of three experiments.

Taken together, these data suggest that vacuoles containing virulent *C. burnetii* follow the endosomal pathway, as revealed by the acquisition of V-H^+-ATPase, Lamp-1, and CD63, but partially acquire M6PR and Rab7.

IFN-γ stimulates bacterial killing, maturation of C. burnetii *vacuoles, and changes of vacuolar pH*

Because IFN-γ induces the killing of virulent *C. burnetii* in THP-1 cells (12), we wondered whether IFN-γ also affects the maturation of *C. burnetii* vacuoles. The addition of IFN-γ (at 200 U/ml) to adherent THP-1 cells before their infection decreased the viability of virulent *C. burnetii* from 92 ± 8% at day 0 to 16 ± 5% after 2 days (Fig. 6). The induction of *C. burnetii* killing was dose-dependent: a concentration of 50 U/ml IFN-γ was sufficient to reduce *C. burnetii* viability (30 and 40% inhibition at days 1 and 2, respectively), and maximum killing (85% inhibition) was obtained with 200 U/ml IFN-γ. We investigated the effect of IFN-γ on intravacuolar pH and colocalization of *C. burnetii* with endosome/lysosome markers. First, IFN-γ significantly ($p < 0.05$) raised the pH of vacuoles containing virulent *C. burnetii* to 6.2 ± 0.3, equivalent to pH values obtained by treating monocytes with bafilomycin A1 (Table I). IFN-γ exerted the same effect on the pH of vacuoles containing avirulent organisms. The alkalinization of bacterial vacuoles occurred after 24 h, suggesting that it was a relatively late event. Second, in the presence of 200 U/ml IFN-γ, the percentage of vacuoles containing virulent *C. burnetii* that colocalized with cathepsin D was 62 ± 6% at h 0 and 80 ± 7% after h 24, whereas it never exceeded 20% in the absence of IFN-γ (Fig. 6). IFN-γ-mediated restoration of bacterial colocalization with cathepsin D was observed with concentrations of IFN-γ similar to those required for bacterial killing (data not shown). However, the cathepsin D that colocalized with virulent organisms was not active. Indeed, the NIRF probe colocalized with virulent organisms

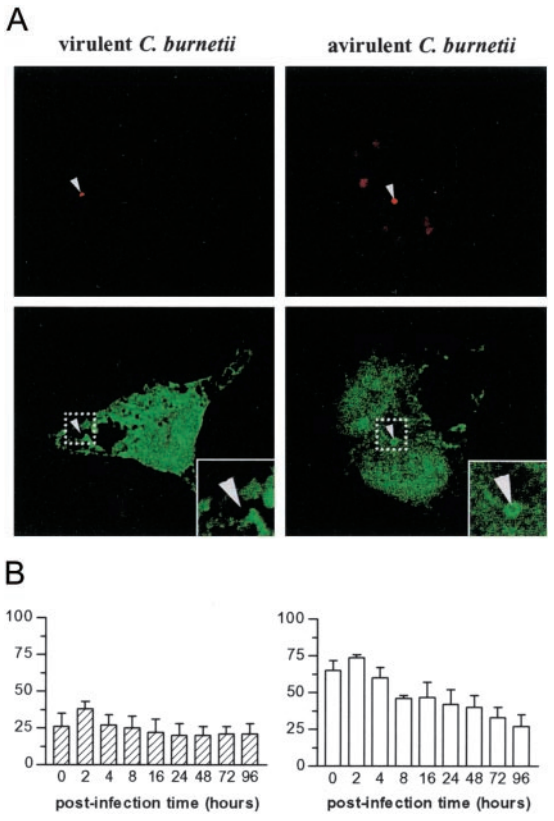

FIGURE 5. Colocalization of *C. burnetii* with Rab7. THP-1 cells were infected by virulent and avirulent *C. burnetii* for 24 h. *A*, Bacteria (*top panels*) and Rab7 (*bottom panels*) were revealed by indirect immunofluorescence and examined by confocal microscopy. Paired photomicrographs are shown. Arrows indicate bacteria (*top panels*) and colocalization with Rab7 (*bottom panels*). Insets show the distribution of Rab7. *B*, Infected cells were incubated again, for 96 h. Results are expressed as the percentage of *C. burnetii* vacuoles expressing Rab7; they are the mean ± SE of three experiments.

as demonstrated by FITC fluorescence, but cathepsin D was inactive as shown by the lack of Cy5.5 fluorescence (Fig. 7). In contrast, the ability of IFN-γ to stimulate the maturation of *C. burnetii* phagosomes involves Rab7. Indeed, IFN-γ increased the colocalization of virulent *C. burnetii* with Rab7 as compared with untreated cells (Fig. 6). The percentage of phagosomes that expressed Rab7 was 53 ± 8% at h 0, and it increased to 83 ± 9% at h 2. After 24 h, 45 ± 10% of phagosomes still expressed Rab7. Taken together, these results show that the pretreatment of THP-1 cells by IFN-γ, which induces *C. burnetii* killing, improves the access of *C. burnetii* phagosomes to some endosomal markers without leading to complete maturation in phagolysosomes.

Adding IFN-γ to infected cells promotes C. burnetii *killing and phagosome maturation without changing the pH*

We wondered whether IFN-γ-induced *C. burnetii* killing results from the acquisition of endosomal/lysosomal markers or the alkalinization of bacterial vacuoles. To discriminate between these two hypotheses, THP-1 cells were infected with *C. burnetii*, thus providing vacuoles containing live organisms, and then were treated with 200 U/ml IFN-γ. This treatment reduced *C. burnetii* viability after 24 and 48 h (72 ± 9 and 38 ± 3% of viable bacteria, respectively), thus confirming the microbicidal effect of IFN-γ administered before *C. burnetii* infection (Fig. 6). Adding IFN-γ to infected cells increased the colocalization of *C. burnetii* with cathepsin D. Indeed, 42 ± 14 and 55 ± 12% of *C. burnetii* vacuoles colocalized with cathepsin D after 8 and 24 h, respectively (Fig. 6). Vacuolar cathepsin D was active as demonstrated by the Cy5.5 fluorescence of the NIRF probe (Fig. 7). In addition, IFN-γ treatment of infected cells restored the colocalization of Rab7 with *C. burnetii* phagosomes as did IFN-γ pretreatment (Fig. 6). In contrast to the effect of IFN-γ pretreatment of THP-1 cells, the addition of IFN-γ to *C. burnetii*-infected cells did not affect the vacuolar pH (Table I). Thus, the effects of IFN-γ on cathepsin D acquisition and vacuolar pH are likely distinct.

Virulent C. burnetii *survives and impairs phagosome-lysosome fusion in circulating monocytes*

To extend the findings we reported in THP-1 cells to circulating monocytes, isolated monocytes were cultured for 3 days to increase their spreading without inducing their maturation into macrophages. This procedure was required to visualize bacterial phagosomes with confocal microscopy. First, we measured the viability of *C. burnetii* in monocytes. The viability of avirulent organisms decreased by 85% after 3 days of culture (Table II) and was residual after 5 days (data not shown). In contrast, the viability

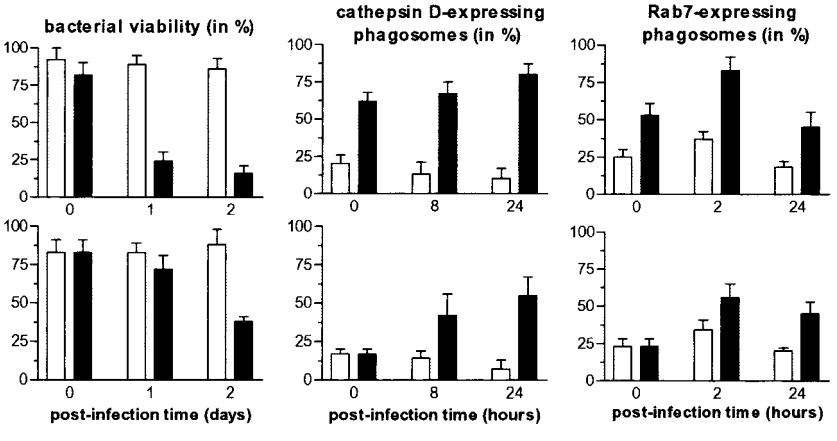

FIGURE 6. Effect of IFN-γ on *C. burnetii* viability and vacuole maturation. THP-1 cells were first incubated with IFN-γ and then infected with virulent *C. burnetii*. After being washed, they were cultured with IFN-γ for different periods (*top panels*). Alternatively, THP-1 cells were first infected by virulent *C. burnetii* and then treated by IFN-γ (*bottom panels*). Bacterial viability was determined, and the results are the mean ± SE of three experiments. Bacteria, cathepsin D, and Rab7 were revealed by indirect immunofluorescence and examined by confocal microscopy. Results are expressed as the percentage of *C. burnetii* vacuoles expressing cathepsin D or Rab7; they are the mean ± SE of three experiments. □, Untreated cells; ■, IFN-γ-treated cells.

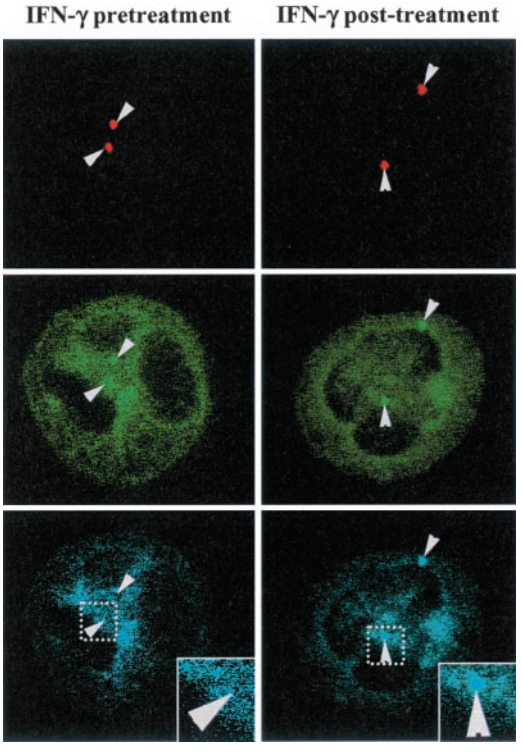

FIGURE 7. Effect of IFN-γ on cathepsin D activity. THP-1 cells were first incubated with IFN-γ and then infected with virulent *C. burnetii*. After being washed, they were cultured with IFN-γ for 24 h (*left panels*). Alternatively, THP-1 cells were first infected by *C. burnetii* and then treated by IFN-γ (*right panels*). NIRF probe (at 0.1 μM) was added to cells for 1 h before the end of the experiment. The colocalization of bacteria (Texas Red fluorescence, *top panels*) with NIRF probe (FITC fluorescence, *middle panels*) was examined with a confocal microscope equipped with appropriate excitation and emission filters. The Cy5.5 fluorescence corresponds to active cathepsin D (*bottom panels*). Arrows indicate bacteria (*top panels*) and NIRF colocalization (*middle* and *bottom panels*). Insets show the colocalization of Cy5.5 fluorescence with *C. burnetii*. The figure is representative of three experiments.

of virulent organisms remained constant during the 5 days of culture. Hence, only virulent *C. burnetii* survived in circulating monocytes, confirming previous results (3, 11). Second, we assessed the colocalization of *C. burnetii* organisms with two markers of phagosome maturation, Lamp-1 and cathepsin D. The percentage of vacuoles that colocalized with Lamp-1 was high in monocytes infected with virulent or avirulent *C. burnetii* at day 0, and all phagosomes had acquired Lamp-1 at day 3 (Table II). In contrast, the pattern of cathepsin D colocalization with *C. burnetii* was different. At day 0, one-third of vacuoles acquired cathepsin D in monocytes infected with avirulent *C. burnetii*, and almost all vacuoles were positive for cathepsin D at day 3. In monocytes infected with virulent organisms, 26 ± 7% of vacuoles colocalized with cathepsin D at day 0, and this percentage remained low even at day 3 (Table II). Taken together, these results show that the survival of virulent *C. burnetii* in circulating monocytes is associated with impaired acquisition of cathepsin D.

Discussion

In this paper, we show that *C. burnetii* escapes killing in THP-1 monocytic cells and circulating monocytes by preventing phagosomal maturation. This finding partly questions the *C. burnetii* paradigm in which *C. burnetii* replicates in the cellular compartment displaying phagolysosomal features (5, 24). The first characteristic reported for *C. burnetii* was that acidic pH is required for bacterial metabolism (6, 7). In agreement with previous reports on avirulent organisms (8, 9), we found *C. burnetii* in acidic vacuoles independently of bacterial virulence. Hence, phagosome pH cannot account for the survival of virulent *C. burnetii* in human monocytes. The second characteristic reported for the *C. burnetii* vacuole is its ability to fuse with different intracellular compartments, including lysosomes. Hence, vacuoles that enclose *C. burnetii* can become large, contain numerous organisms, and fuse with vacuoles containing *Leishmania amazonensis* or *M. avium* (25, 26). The conclusions of these studies are limited by their use of avirulent organisms and cells in which *C. burnetii* replication was independent of bacterial virulence. We show that vacuoles containing virulent *C. burnetii* did not acquire cathepsin D, a lysosomal hydrolase. The impairment of cathepsin D accumulation reflects defective phagolysosomal fusion because lysosomal trackers did not accumulate in *C. burnetii* phagosomes. The lack of colocalization of *C. burnetii* and cathepsin D was not due to delayed acquisition of cathepsin D by *C. burnetii* vacuoles, which disagrees with a recent paper in which virulent *C. burnetii* delays phagolysosomal fusion in J774 cells (27), but these murine macrophage-like cells allow the replication of both virulent and avirulent organisms. In blood monocytes, in which only virulent *C. burnetii* organisms survive (Refs. 3 and 11 and our results), bacterial phagosomes were unable to fuse with lysosomes. This was specific of bacterial virulence because cathepsin D accumulated within vacuoles containing avirulent *C. burnetii*. In addition, two other virulent strains of *C. burnetii* exhibit similar impairment of phagosome-lysosome fusion. The lack of cathepsin D colocalization with *C. burnetii* did not result from impaired interactions of bacterial vacuoles with the endocytic pathway. Hence, markers of late endosomes and early lysosomes such as Lamp-1, CD63, V-H^+-ATPase were acquired by *C. burnetii* vacuoles independently of bacterial virulence. Thus, the survival of *C. burnetii* in monocytic cells is associated with altered phagosomal maturation, which is reminiscent of the escape mechanism used by *Salmonella enterica*. Indeed, both types of pathogen-containing vacuoles acquire markers of late endosomes-early lysosomes such as Lamp-1, but are devoid of lysosomal enzymes (28, 29). However, the molecular mechanisms involved in the control of phagosome maturation are likely different. *C. burnetii*

Table II. *Survival and intracellular traffic of* C. burnetii *in circulating monocytes*[a]

	Virulent *C. burnetii*		Avirulent *C. burnetii*	
	Day 0 (%)	Day 3 (%)	Day 0 (%)	Day 3 (%)
Bacterial viability	72 ± 4	82 ± 5	63 ± 6	15 ± 10
Lamp-1 vacuoles	64 ± 10	100	70 ± 6	100
Cathepsin D vacuoles	26 ± 7	17 ± 6	33 ± 9	92 ± 6

[a] Circulating monocytes were cultured for 3 days and incubated with *C. burnetii* for 24 h (day 0) and then incubated for 72 additional hours (day 3). The bacterial viability was determined using SYTO9 and propidium iodide. The results are expressed in percentage of viable bacteria. *C. burnetii* organisms, Lamp-1 and cathepsin D were revealed by immunofluorescence, and their colocalization was analyzed by confocal microscopy. Results are expressed as the percentage of *C. burnetii* vacuoles expressing Lamp-1 or cathepsin D; they are the mean ± SE of four experiments.

vacuoles partly acquired the late-endosomal GTPase Rab7, while Salmonella vacuoles recruit Lamp-1 in a Rab7-dependent manner (30). It is likely that the ability of Rab7 to regulate vesicle traffic in late endocytosis (31, 32) is altered in *C. burnetii* infection. This hypothesis is strengthened by the finding that *C. burnetii* had no effect on the early acquisition of EEA1, a marker of early endosomes (data not shown). The strategy of *C. burnetii* survival in human monocytes is likely based on interference with Rab7 that controls transport to endocytic degradative compartments, leading to the formation of a vacuole unable to fuse with lysosomes.

IFN-γ stimulated the killing of *C. burnetii* by THP-1 cells and affected the maturation of *C. burnetii* vacuoles as assessed by the acquisition of cathepsin D. IFN-γ likely affects cathepsin D acquisition by distinct mechanisms. IFN-γ pretreatment of THP-1 cells induced the accumulation of inactive cathepsin D by *C. burnetii* vacuoles, which is reminiscent of the results of Ullrich et al. (33), who found that *M. avium* phagosomes acquire an inactive form of cathepsin D. IFN-γ also stimulated the alkalinization of *C. burnetii* vacuoles. Vacuole alkalinization did not result from the exclusion of V-H^+-ATPase because the colocalization of V-H^+-ATPase and *C. burnetii* was similar in THP-1 cells treated or not with IFN-γ. This finding is surprising because IFN-γ has been reported to lower the pH of *M. avium* vacuoles through the accumulation of V-H^+-ATPase (14) and to impair the interaction of phagosomes with late endosomes and lysosomes without interfering with acidification (16). In contrast, adding IFN-γ to infected THP-1 cells stimulated the acquisition of active cathepsin D but it had no effect on vacuolar pH. Thus, it is likely that IFN-γ-induced phagolysosomal fusion and vacuolar alkalinization play different roles in *C. burnetii* killing. These results have pathophysiological consequences. Resting monocytic cells are unable to kill virulent *C. burnetii* but cannot support bacterial replication. This latter is only achieved when monocytes are specifically deactivated by IL-10 (11). IL-10 does not modify the traffic of *C. burnetii* vacuoles in monocytes (our unpublished data). In contrast, the activation of monocytic cells by IFN-γ reprogrammed them to be microbicidal against *C. burnetii* through phagosomal maturation. As IFN-γ is associated with the cure of *C. burnetii* infections (2), it is likely that the restoration of phagosome-lysosome fusion is critical for the control of Q fever.

The survival of *C. burnetii* into THP-1 cells and monocytes is associated with altered phagosome maturation. The activation of these professional phagocytes by IFN-γ leads to *C. burnetii* killing and restores phagosomal maturation. We propose two potential mechanisms for IFN-γ-induced killing of *C. burnetii*: an early mechanism based on phagosome maturation and a late mechanism involving modulation of vacuolar pH. Therapeutic elimination of *C. burnetii* in Q fever might benefit from exploring these two parameters of bacterial killing.

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