Burkholderia cenocepacia-induced delay of acidification and phagolysosomal fusion in cystic fibrosis transmembrane conductance regulator (CFTR)-defective macrophages

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The *Burkholderia cepacia* complex (Bcc) is a group of opportunistic bacteria chronically infecting the airways of patients with cystic fibrosis (CF). Several laboratories have shown that Bcc members, in particular *B. cenocepacia*, survive within a membrane-bound vacuole inside phagocytic and epithelial cells. We have previously demonstrated that intracellular *B. cenocepacia* causes a delay in phagosomal maturation, as revealed by impaired acidification and slow accumulation of the late phagolysosomal marker LAMP-1. In this study, we demonstrate that uninfected cystic fibrosis transmembrane conductance regulator (CFTR)-defective macrophages or normal macrophages treated with a CFTR-specific drug inhibitor display normal acidification. However, after ingestion of *B. cenocepacia*, acidification and phagolysosomal fusion of the bacteria-containing vacuoles occur in a lower percentage of CFTR-negative macrophages than CFTR-positive cells, suggesting that loss of CFTR function contributes to enhance bacterial intracellular survival. The CFTR-associated phagosomal maturation defect was absent in macrophages exposed to heat-inactivated *B. cenocepacia* and macrophages infected with a non-CF pathogen such as *Salmonella enterica*, an intracellular pathogen that once internalized rapidly traffics to acidic compartments that acquire lysosomal markers. These results suggest that not only a defective CFTR but also viable *B. cenocepacia* are required for the altered trafficking phenotype. We conclude that CFTR may play a role in the mechanism of clearance of the intracellular infection, as we have shown before that *B. cenocepacia* cells localized to the lysosome lose cell envelope integrity. Therefore, the prolonged maturation arrest of the vacuoles containing *B. cenocepacia* within *cftr*^{-/-} macrophages could be a contributing factor in the persistence of the bacteria within CF patients.

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INTRODUCTION

Cystic fibrosis (CF) is a lethal recessive human genetic disorder caused by mutations in the gene encoding a cAMP-regulated chloride channel (cystic fibrosis transmembrane conductance regulator; CFTR) that is predominantly expressed on the apical membrane of epithelial cells (Boucher, 2007; Cheng *et al.*, 1990; Riordan *et al.*, 1989). The airways of CF patients become chronically infected with a restricted subset of opportunistic soil bacteria belonging to the *Pseudomonas* and *Burkholderia* genera (Govan *et al.*, 1996; Govan & Deretic, 1996). Chronic infection leads to

progressive airway tissue destruction, pulmonary dysfunction, and death. *Burkholderia cepacia* complex (Bcc) strains have become important human opportunistic pathogens (Coenye & Vandamme, 2003; Mahenthiralingam *et al.*, 2005), particularly in young adult patients with CF. *B. cenocepacia* is one of the most prevalent and pervasive Bcc species in Canada and other parts of the world (Speert *et al.*, 2002). Bcc and non-Bcc species of *Burkholderia* are characterized by their metabolic diversity and ability to adapt to varying environmental conditions, including nutrient limitation, antibiotics, antimicrobial peptides and toxic compounds (Coenye & Vandamme, 2003).

Survival and persistence within host cells and tissues are believed to play a key role in the adaptation of *Burkholderia* species to multiple niches, including roots and other plant tissues, free-living cells in the environment, and humans.

Abbreviations: Bcc, *Burkholderia cepacia* complex; BcCV, *Burkholderia cenocepacia*-containing vacuole; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; eGFP, enhanced green-fluorescent protein; mRFP1, monomeric red-fluorescent protein 1; TMR-dextran, tetramethylrhodamine-dextran.

Indeed, *Burkholderia* species can be found as obligate intracellular endosymbionts in pathogenic fungi (Partida-Martinez & Hertweck, 2005), as symbiotic and endophytic bacteria inhabiting leaf galls, roots and other tissues of plants (Balandreau & Mavingui, 2006; Van Oevelen *et al.*, 2002), and within free-living amoebae (Inglis *et al.*, 2000; Lamothe *et al.*, 2004; Landers *et al.*, 2000; Marolda *et al.*, 1999). Direct evidence of *ex vivo* intracellular survival of Bcc isolates in mammalian cells has been demonstrated in the human respiratory epithelial cell line A549 and in cultures of primary lung epithelial cells (Burns *et al.*, 1996; Keig *et al.*, 2001, 2002; Sajjan *et al.*, 2006). Although it is unclear whether intracellular survival in epithelial cells is relevant *in vivo*, the capacity of Bcc strains to penetrate epithelial cells correlates with mouse infectivity (Chiu *et al.*, 2001; Cieri *et al.*, 2002). Furthermore, *Burkholderia pseudomallei* (Jones *et al.*, 1996) and *Burkholderia mallei* (Ribot & Ulrich, 2006) survive and replicate in macrophage cell lines, unlike Bcc strains, which survive with minimal or no replication (Lamothe *et al.*, 2007; Martin & Mohr, 2000; Saini *et al.*, 1999). These differences may be due to the ability of *B. mallei* and *B. pseudomallei* to escape from the phagocytic vacuole shortly after phagocytosis and replicate in the cytosol, a process that requires bacterial effectors secreted by type III secretion systems (Stevens *et al.*, 2002, 2003). In contrast, phagocytized Bcc strains reside in bacteria-containing vacuoles (BcCVs) that at least for the initial hours post-infection do not acidify and avoid fusion with lysosomes by a type III-independent mechanism (Lamothe *et al.*, 2007). The bacterial determinants and the host cell targets involved in the mechanism underlying Bcc-mediated altered phagosome maturation still remain unknown.

The ability of Bcc strains to colonize and infect the mucosal airways of CF patients is not well understood. Recent work has suggested that phagosomes of CFTR-defective alveolar macrophages and neutrophils exhibit a constitutive acidification delay that could contribute to alveolar macrophage dysfunction, presumably facilitating infection by CF-related pathogens such as *Pseudomonas aeruginosa* (Di *et al.*, 2006). However, an independent study using fluorescence ratio imaging to measure the endosomal pH against an internal standard unequivocally demonstrated that phagolysosomal acidification in alveolar macrophages is CFTR-independent (Haggie & Verkman, 2007), although these investigators did not examine directly the ability of CFTR macrophages to clear an intracellular infection. In agreement with the latter study, we demonstrate here that uninfected CFTR-defective macrophages or normal macrophages treated with a CFTR-specific inhibitor display normal acidification. However, following infection with *B. cenocepacia*, BcCVs from CFTR-defective macrophages exhibit a more pronounced delay in acidification and phagolysosomal fusion than that observed with normal macrophages. The CFTR-associated phagosomal maturation defect was absent in macrophages exposed to heat-inactivated *B. cenocepacia* and macrophages infected with a non-CF pathogen such as *Salmonella enterica*, a bacterium that once it becomes intracellular rapidly traffics

to acidic compartments that acquire lysosomal markers. We conclude that CFTR somehow contributes to the clearance of the intracellular infection and further maturation of the BcCVs, but that this process is specific for *B. cenocepacia* and perhaps other pathogens that chronically infect the airways of CF patients.

METHODS

Reagents and antibodies. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Wisent. Dextran tetramethylrhodamine, dextran Oregon green 514 and dextran FITC (molecular mass 10 000 Da) were from Molecular Probes. The primary monoclonal rat anti-mouse LAMP-1 (ID4B) antibody was from the Developmental Studies Hybridoma Bank. Polyclonal goat anti-mouse EEA1 (IgG) was from Santa Cruz Biotechnology. The secondary Alexa 488-conjugated chicken anti-rat and chicken anti-goat antibodies were purchased from Molecular Probes. Concanamycin A and CFTR_{inh}-172 were from Sigma.

Bacterial strains, macrophages and culture conditions. *B. cenocepacia* J2315 is a prototypic strain of the highly transmissible ET12 clone (Govan *et al.*, 1993; Johnson *et al.*, 1994). *S. enterica* serovar Typhimurium LT2 was from our culture collection. Bacteria were grown at 37 °C in Luria-Bertani (LB) medium with agitation. *B. cenocepacia* carrying the arabinose-inducible plasmid pMLBAD-eGFP (Lefebvre & Valvano, 2002), which expresses the enhanced green-fluorescent protein (eGFP), was grown with 2% (w/v) arabinose and a final concentration of 100 µg trimethoprim ml⁻¹. *B. cenocepacia* carrying pJR1 (Lamothe *et al.*, 2007), which expresses the monomeric red-fluorescent protein 1 (mRFP1), was grown in the presence of 100 µg trimethoprim ml⁻¹, and *S. enterica* carrying pRSETb-mRFP1 (Campbell *et al.*, 2002) was grown in the presence of 100 µg ampicillin ml⁻¹. RAW 264.7 macrophages were derived from BALB/c mice (TIB-71, ATCC). The murine *cfr*^{-/-} and *cfr*^{+/-} macrophage cell lines were obtained from Dr D. Radzioch, McGill University. Bone marrow-derived macrophages from the CF-*neo* ablation were generated by stable transfection of the retroviral recombinant J2 construct expressing part of the *v-raf* and *v-myc* genes, as described previously (Blasi *et al.*, 1989). The cell lines were derived from the bone marrow of littermate control mice (born from the same carrier of the CF-*neo* ablation vector parents). The *cfr* gene knockout mice and their littermate *cfr* parental controls were on the same C57BL/6 genetic background. The ablation of the *cfr* gene in the *cfr*^{-/-} macrophages was confirmed in our laboratory by PCR amplification of an internal fragment of the murine *cfr* gene exon 10 using primers CFEx10NT (5'-GGAATTATTAAGCACAGTGGAGAG-3') and CFEx10CT (5'-GCTTTACTGGAGAAAGTAAGCTACTAG-3'). Cells were maintained in DMEM with 10% FBS and grown at 37 °C in a humidified atmosphere under 5% carbon dioxide.

Macrophage infection assays. Bacterial strains were washed twice and resuspended in DMEM 10% FBS. Heat-inactivated bacteria were obtained by incubation at 60 °C for 25 min prior to infection. Under these conditions we have noticed 100% bacterial cell death upon plating in Luria broth medium. Bacteria were added to macrophage cells grown on glass coverslips at the m.o.i. indicated in each experiment, centrifuged for 1 min at 300 g and incubated at 37 °C with 5% carbon dioxide. Infected macrophages were washed three times with 1 × PBS (Wisent) and observed under a fluorescence microscope or reincubated at 37 °C under 5% carbon dioxide in DMEM containing 10% FBS. In a previous study (Lamothe *et al.*, 2007), we have investigated the internalization of *B. cenocepacia* strain J2315 in the mouse macrophage-like cell line RAW 264.7, and determined that an m.o.i. of 30–50 allows us to find a reasonable proportion of infected macrophages with a sufficient number of

intracellular bacteria for analysis. Therefore, unless otherwise indicated, all the experiments in this study were performed using an m.o.i. of 30.

Live fluorescence microscopy. For lysosome labelling, macrophages were incubated at 37 °C with 5 % carbon dioxide in the presence of 250 $\mu\text{g ml}^{-1}$ tetramethylrhodamine-dextran (TMR-dextran) for 2 h and chased for 1 h in DMEM with 10 % FBS. Labelling of the endosomal pathway was done by incubating macrophages with *B. cenocepacia* J2315 for 2 h, followed by a 1 h chase to allow maturation of the BcCVs, and a final incubation with 250 $\mu\text{g ml}^{-1}$ TMR-dextran for an additional 2 h. External TMR-dextran was removed by serial washes with PBS and the macrophages were immediately visualized. Images were acquired using an Axioscope 2 (Carl Zeiss) microscope with a $\times 100$ oil immersion objective coupled to a Qimaging (Burnaby) cooled charged-coupled device camera.

Immunofluorescence and confocal microscopy. Cells were fixed in 4 % paraformaldehyde (v/v) for 30 min at room temperature and incubated for 10 min with 100 mM glycine in $1 \times$ PBS. Cells were then permeabilized with 0.1 % Triton X-100 (v/v) and blocked with 5 % milk powder (w/v) for 1 h at room temperature. Permeabilized cells were incubated with primary antibodies, followed by secondary antibodies in 5 % milk powder for 1 h each at room temperature. Coverslips were mounted onto glass slides using fluorescent mounting medium (DakoCytomation). All secondary antibodies were used at a dilution of 1:1000, rat anti-LAMP-1 at 1:50 and goat anti-EEA1 at 1:100. Confocal microscopy was performed using a Zeiss LSM 510 laser scanning confocal microscope and a $\times 100$ oil immersion objective. In each experiment, the colocalization of LAMP-1 and the BcCVs was determined by examining 21 fields at $\times 100$ and counting the number of BcCVs surrounded by a ring-like structure per macrophage cell.

Analysis of endosomal pH. Macrophages were grown on coverslips in DMEM with 10 % FBS to a confluence of 60 %. Monolayers were then incubated simultaneously with 250 $\mu\text{g ml}^{-1}$ TMR-dextran and 250 $\mu\text{g ml}^{-1}$ FITC-conjugated dextran for 2 h or overnight at 37 °C. External dextran was removed by three washes of 2 ml PBS and macrophages were reincubated for 2 h in DMEM 10 % FBS to chase dyes to lysosome compartments. In control experiments, macrophages were preincubated for 20 min with 250 nM concanamycin (to inhibit the activity of the vacuolar ATPase; Whyteside *et al.*, 2005) or 10 μM CFTR_{inh}-172, a thiazolidinone compound that specifically inhibits CFTR ion channel activity and has no other known toxic effects for rodent cells in culture (Ma *et al.*, 2002; Taddei *et al.*, 2004). To activate macrophages, monolayers were incubated with 500 ng ml^{-1} of *B. cenocepacia* J2315 LPS. Pictures were taken using a Zeiss LSM 510 laser scanning confocal microscope with a $\times 100$ oil immersion objective, and laser power remained constant between each experiment. Fluorescence intensities were analysed using LSM Image Examiner software and presented as fluorescence ratios (FITC/TMR).

Statistical analyses. The statistical analysis was done using GraphPad Prism version 4.03 (GraphPad Software) by two-way ANOVA and Student's *t* test, depending on the experiment. Data were collected by counting the BcCVs per macrophage cell over 21 fields in triplicate.

RESULTS AND DISCUSSION

CFTR dysfunction does not alter the early maturation of BcCVs

To characterize the nature of the compartment occupied by intracellular *B. cenocepacia* in macrophages with altered CFTR function we used three murine macrophage cell

lines: RAW 264.7 macrophages, derived from BALB/c mice, and a pair of congenic *cftr*^{+/+} and *cftr*^{-/-} macrophage cell lines derived from C57BL/6 mice. CFTR function in RAW 264.7 macrophages was pharmacologically abrogated by treatment with CFTR_{inh}-172. We performed all of these experiments with *B. cenocepacia* strain J2315, the index case clinical isolate of the ET12 epidemic clone that has been associated with severe lung deterioration in CF patients (Govan *et al.*, 1993). Previously, our laboratory demonstrated that strain J2315 is poorly internalized by RAW 264.7 macrophages. However, m.o.i. values of 30–50 result in a reasonable proportion of infected macrophages and are sufficient for microscopic analysis (Lamothe *et al.*, 2007). Also, in previous work, we have shown that the phagosomes of RAW 264.7 macrophages containing live *B. cenocepacia* J2315 remain functionally connected to the endocytic pathway, as revealed by the continued access of endocytic tracers, such as fluorescent dextran, to the BcCVs (Lamothe *et al.*, 2007). It should be noted that lack of intracellular bacterial replication and the failure to kill extracellular bacteria prevent us from obtaining viable counts in macrophage infection experiments (Lamothe *et al.*, 2007; Saini *et al.*, 1999). Therefore, to examine the effect of the CFTR status on the intracellular behaviour of *B. cenocepacia*, we conducted single-cell analyses using a variety of markers that correspond to various stages of phagosomal maturation. We first investigated whether BcCVs in macrophages devoid of CFTR function also remain connected with the endocytic pathway. Macrophages were incubated with live *B. cenocepacia* for 2 h, allowed to mature for 1 h and then incubated with TMR-dextran (see Methods). BcCVs in both *cftr*^{+/+} and *cftr*^{-/-} macrophages, as well as BcCVs from infected RAW 264.7 macrophages that were pre-incubated with CFTR_{inh}-172, incorporated fluorescent dextran (Fig. 1a). Quantitative analysis demonstrated that on average 75 % of BcCVs accumulated external dextran with no statistically significant differences among the cell lines, irrespective of CFTR function (Fig. 1b). Therefore, the CFTR functional status does not affect the interactions of BcCVs with newly formed endocytic vacuoles. We also demonstrated previously that the EEA1 protein, a marker for the sorting vesicles of the endocytic pathway, is transiently recruited within minutes to BcCVs but dissociates from these vacuoles after 30–45 min (Lamothe *et al.*, 2007). At 30 min post-infection, EEA1 colocalized with BcCVs in both *cftr*^{+/+} and *cftr*^{-/-} macrophages (Fig. 2), but as expected, this marker disappeared at later incubation times. Together, these experiments demonstrate that CFTR dysfunction does not alter the early maturation events of the BcCVs.

A dysfunctional CFTR enhances the *B. cenocepacia*-mediated maturation defect of the BcCVs

In RAW 264.7 macrophages infected with *B. cenocepacia* there is a delay of up to 6 h before the late phagosome/

Fig. 1. BcCVs remain functionally associated with incoming endosomes. (a) eGFP fluorescent *B. cenocepacia* J2315, TMR-dextran and merged pictures of BcCVs from *cftr*^{+/+} macrophages (top panels), *cftr*^{-/-} macrophages (middle panels) and RAW 264.7 macrophages exposed to 10 μ M of CFTR^{inh}-172 (bottom panels). All experiments were conducted at an m.o.i. of 30, which we have previously determined to be optimal conditions for phagocytosis of *B. cenocepacia* (Lamothe *et al.*, 2007). Bar, 11 μ m. (b) Percentage of BcCVs accumulating external dextran in *cftr*^{+/+} (white bar), *cftr*^{-/-} (black bar) and CFTR^{inh}-172-treated (hatched bar) RAW 264.7 macrophages. The values represent the mean and SD of three experiments in which 21 fields were examined at $\times 100$. Error bars, SEM.

Fig. 2. BcCVs transiently accumulate the early endosomal marker EEA1. *cftr*^{+/+} (top panels) and *cftr*^{-/-} (bottom panels) macrophages were infected with live *B. cenocepacia* J2315 expressing mRFP1 (red fluorescence) at an m.o.i. of 50. Immunolabelling was performed using goat anti-EEA1 and a secondary anti-goat antibody conjugated to AlexaFluor 488 (green fluorescence). Images were captured with a 510 LSM confocal microscope and $\times 100$ objective. Bar, 6 μ m.

lysosome marker LAMP-1 is clearly detected in the membrane surrounding BcCVs, forming a typical ring-like fluorescent structure (Lamothe *et al.*, 2007). In contrast, at earlier time points, there is a redistribution of the LAMP-1-rich compartment, as demonstrated by images showing clumps of LAMP-1 fluorescence surrounding intracellular bacteria with no typical ring formation (Lamothe *et al.*, 2007). We investigated whether CFTR function is required for this late maturation step by monitoring the temporal accumulation of LAMP-1 into BcCVs of CFTR-defective macrophages. As expected according to our previous study (Lamothe *et al.*, 2007), BcCVs of CFTR_{inh}-172-treated and -untreated RAW 264.7 cells, as well as BcCVs of *cftr*^{+/+} and *cftr*^{-/-} macrophages, did not accumulate LAMP-1 between 1 and 4 h post-infection. However, at 8 h post-infection, 80 ± 2 % of BcCVs from *cftr*^{+/+} macrophages acquired LAMP-1, giving rise to typical ring-like structures (Fig. 3a, top-middle panel, arrow-

heads, and Fig. 3b). In contrast, rings of LAMP-1-positive material were observed in fewer than 32 ± 3 % of BcCVs in *cftr*^{-/-} cells ($P < 0.001$; Fig. 3a, centre panel, and Fig. 3b). At 8 h post-infection, several BcCVs in *cftr*^{+/+} macrophages also demonstrated a uniform red fluorescent background (Fig. 3a, top-left panel, arrows). We have previously shown that the escape of mRFP1 from the bacterial cytoplasm to the lumen of the BcCVs occurs after phagolysosomal fusion (Lamothe *et al.*, 2007), indicating loss of bacterial cell envelope integrity. This escape of mRFP1 was not observed in BcCVs of infected *cftr*^{-/-} macrophages (Fig. 3a, middle-left panel), suggesting that bacteria in these vesicles still have an intact cell envelope. RAW 264.7 macrophages incubated with CFTR_{inh}-172 also did not reveal a significant number of typical rings of LAMP-1 colocalization with BcCVs at 8 h post-infection (Fig. 3b). However, clumps of LAMP-1-positive material were found colocalizing with BcCVs (Fig. 3a, lower panels). We have previously observed this

Fig. 3. Colocalization of LAMP-1 to BcCVs. (a) Confocal images of *cftr*^{+/+}, *cftr*^{-/-} and CFTR_{inh}-172-treated RAW 264.7 macrophages infected with *B. cenocepacia* expressing mRFP1 (red fluorescence) at 8 h post-infection and at an m.o.i. of 30. Immunolabelling was performed using rat anti-LAMP-1 and a secondary anti-rat antibody conjugated to AlexaFluor 488 (green fluorescence). Arrowheads in the top-middle panel point to typical ring-like structures indicating the colocalization of LAMP-1 with the BcCVs, which is only seen in *cftr*^{+/+}-infected macrophages. Arrows in the upper- and lower-left panels point to areas of diffuse red fluorescence, which are likely due to the release of mRFP1 content from the bacterial cytoplasm after phagolysosomal fusion (Lamothe *et al.*, 2007). Images were captured with a 510 LSM confocal microscope and a ×100 objective. Bar, 10 µm. (b) Quantitative analysis of LAMP-1 ring formation over the infection time in *cftr*^{+/+} (white bar), *cftr*^{-/-} (black bar) and CFTR_{inh}-172-treated RAW 264.7 (hatched bar) macrophages. Bars represent the mean percentage and SEM from infection experiments in which 21 fields were examined at ×100 for formation of LAMP-1 fluorescent rings around the BcCVs. ***The percentage of LAMP-1 accumulation onto BcCVs of *cftr*^{+/+}-infected cells at 6 and 8 h post-infection was significantly higher than in *cftr*^{-/-} cells or in RAW 264.7 macrophages treated with CFTR_{inh}-172 ($P < 0.001$). Pair-wise comparisons at other times were non-significant.

phenomenon in untreated RAW 264.7 macrophages infected with live *B. cenocepacia* just prior to ring formation (Lamothe *et al.*, 2007), but at 1–3 h post-infection. We also observed in some of the BcCVs the release of mRFP1 to the vacuolar lumen (Fig. 3a, lower-left panel, arrows), suggesting that phagolysosomal fusion had begun to take place. Therefore, these results suggest that in the presence of CFTR_{inh}-172 there is also a significant delay in the maturation of the BcCVs, albeit less pronounced than that found with *cftr*^{-/-} macrophages.

The pronounced maturation delay observed with *cftr*^{-/-} and CFTR_{inh}-172-treated RAW 264.7 macrophages, as judged by the ability of BcCVs to accumulate LAMP-1, was not noticeable when these cells were exposed to heat-inactivated *B. cenocepacia*. Indeed, BcCVs carrying heat-inactivated *B. cenocepacia* rapidly fused with LAMP-1-rich compartments at 30 min post-infection in the three cell types tested (Fig. 4, see arrowheads). Quantitative analysis revealed that on average 80 % of BcCVs colocalized with LAMP-1-positive material forming ring-like structures in all of the cell types. Therefore, BcCVs containing nonviable *B. cenocepacia* in *cftr*^{-/-} macrophages or in CFTR-inhibited cells trafficked normally into LAMP-1-rich vacuoles, as we have previously found in normal RAW 264.7 macrophages (Lamothe *et al.*, 2007). These results argue that the absence of CFTR expression in *cftr*^{-/-} cells or the pharmacological abrogation of CFTR function by CFTR_{inh}-172 exaggerates the *B. cenocepacia*-mediated traffic delay of the BcCVs into the lysosomes.

A dysfunctional CFTR has no impact on the maturation of vacuoles containing *S. enterica*

To assess whether the absence of a functional CFTR protein has any effect on the trafficking of other intracellular bacteria, we infected *cftr*^{-/-} macrophages with *S. enterica* strain LT2. *Salmonella* is a bona fide intracellular pathogen that promotes its own uptake, and since it has been shown that a high bacterial inoculum may lead to macrophage cell death induced by intracellular *Salmonella* (Fink & Cookson, 2007; Monack *et al.*, 1996; Rupper & Cardelli, 2008), we used a lower m.o.i. for these experiments. It has been reported that the bacteria-containing vacuoles of RAW 264.7 cells infected with *S. enterica* acquire LAMP-1 relatively early after infection (Beuzón *et al.*, 2000; Rathman *et al.*, 1997). At 4 h post-infection, phagosomes containing live *S. enterica* from *cftr*^{-/-} and RAW 264.7 cells incubated with CFTR_{inh}-172 accumulated LAMP-1 (Fig. 5). These results indicate that the delay in LAMP-1 accumulation is specific to the vacuoles containing live *B. cenocepacia* and is not a general defect with any viable intracellular bacteria.

Uninfected CFTR-defective macrophages display normal phagolysosomal acidification

Internalized *B. cenocepacia* within macrophages reside in poorly acidified vacuoles before fusion with lysosomes (Lamothe *et al.*, 2007). To assess the state of acidification of vacuoles carrying live *B. cenocepacia* in *cftr*^{-/-} macrophages, infected cells were incubated with LysoTracker Red (Invitrogen). This fluorescent probe accumulates preferentially within the most acidic compartments of the cell due to

Fig. 4. Vacuoles containing heat-inactivated *B. cenocepacia* rapidly accumulate LAMP-1 in the absence of CFTR function. *cftr*^{+/+}, *cftr*^{-/-} and CFTR_{inh}-172-treated RAW 264.7 macrophages were infected with heat-inactivated *B. cenocepacia* expressing mRFP1 (red fluorescence) at an m.o.i. of 30 and fixed at 30 min post-infection. Immunolabelling was performed using rat anti-LAMP-1 and a secondary anti-rat antibody conjugated to AlexaFluor 488 (green fluorescence). Arrowheads point to typical ring-like structures denoting the colocalization of LAMP-1 with the BcCVs, which is observed in the three types of macrophages. Images were captured with a 510 LSM confocal microscope and ×100 objective. Bar, 12 μm.

Fig. 5. Colocalization of LAMP-1 with *S. enterica*-containing vacuoles in *cftr*^{-/-} and CFTR_{inh}-172-treated RAW 264.7 macrophages. Murine macrophages were infected with live *S. enterica* LT2 expressing mRFP1 (red fluorescence) at an m.o.i. of 10 and fixed at 4 h post-infection. Immunolabelling was performed using rat anti-LAMP-1 and a secondary anti-rat antibody conjugated with AlexaFluor 488 (green fluorescence). Arrowheads point to typical ring-like structures denoting the colocalization of LAMP-1 with the *Salmonella*-containing vacuoles. Images were captured with a 510 LSM confocal microscope and ×100 objective. Bar, 6 µm.

the formation of a membrane-impermeable form upon protonation in low pH environments (Lemieux *et al.*, 2004). The accumulation of LysoTracker into BcCVs of *cftr*^{+/+} and *cftr*^{-/-} macrophages infected with live *B. cenocepacia* was quantitatively assessed 8 h after internalization by counting BcCVs in 21 fields in triplicate experiments. At 8 h post-infection, 23 ± 4% of BcCVs within *cftr*^{-/-} macrophages colocalized with the acidic probe compared with 83 ± 3% of BcCVs within *cftr*^{+/+} macrophages ($P < 0.001$; data not shown). These results demonstrate that the vacuoles containing *B. cenocepacia* in *cftr*^{-/-} macrophages remain poorly acidified at a time that corresponds to the extended delay in BcCV maturation. Consistent with poor acidification and the absence of LAMP-1 localization to the BcCVs, vacuoles carrying live *B. cenocepacia* in *cftr*^{-/-} macrophages did not colocalize with dextran-labelled lysosomes (data not shown).

It has recently been suggested that CFTR plays a role in the pH regulation of alveolar macrophage lysosomes (Di *et al.*, 2006). However, others have observed no pH variation in organelles of cells that lack CFTR function (Gibson *et al.*, 2000; Haggie & Verkman, 2007). Since acidification of endosomes is a critical factor for phagosomal maturation (Clague *et al.*, 1994; van Weert *et al.*, 1995) and LAMPs are required for lysosomal fusion (Huynh *et al.*, 2007), we investigated the relationship between CFTR and lysosome acidification in our cell lines. Ratiometric fluorescence analyses between pH-insensitive (TMR-dextran) and pH-sensitive (FITC-dextran) dyes were performed to evaluate acidification of lysosome-like compartments in both resting and activated macrophages. Macrophages from *cftr*^{-/-} and *cftr*^{+/+} genetic backgrounds were incubated with pH-sensitive and pH-insensitive fluorescently labelled dextrans (see Methods) and fluorescence ratios (FITC/TMR) were compared over time. As controls, fluorescence ratios were determined after treatment with CFTR_{inh}-172 and concanamycin A, a V-H⁺ ATPase inhibitor (Whyteside *et al.*, 2005). FITC fluorescence in the

endosomal compartments from both *cftr*^{-/-} and *cftr*^{+/+} macrophages was quenched, indicating luminal acidification, but the endosomes remained strongly fluorescent in cells pre-treated with concanamycin A (Fig. 6a). The fluorescence ratios (FITC/TMR) of concanamycin A-treated cells were around 1, while the fluorescence ratios of *cftr*^{-/-}, *cftr*^{+/+} and RAW 264.7 macrophages treated with the CFTR inhibitor averaged between 0.6 and 0.7 (Fig. 6b), demonstrating no statistically significant differences between the three experiments. It could be argued that one major confounding factor in our experiments is the presence of extracellular bacteria during the entire course of the experiment. However, similar results were also obtained from radiometric analyses using macrophages that were activated by treatment with 500 ng *B. cenocepacia* J2315 LPS (data not shown), a condition that leads to macrophage cell activation by stimulating the synthesis and secretion of tumour necrosis factor-1 and the oxidative burst (Saini *et al.*, 1999; K. Keith and M. A. Valvano, unpublished results). Together, these data demonstrate that in agreement with an earlier report (Haggie & Verkman, 2007), uninfected *cftr*^{-/-} macrophages or macrophages treated with CFTR_{inh}-172 do not have an intrinsic defect in their ability to acidify lysosomal compartments. It has been shown elsewhere that the CFTR protein in macrophages and neutrophils can be visualized in the membrane of vacuoles (Di *et al.*, 2006; Painter *et al.*, 2006). Experiments in our laboratory using five different CFTR-specific antibodies, including the one used in an earlier report (Di *et al.*, 2006), did not yield any results. However, CFTR expression was detected by RT-PCR (data not shown). These results support the notion that CFTR is present in these cells at very low levels of expression.

Concluding remarks

Macrophages are increasingly recognized as having a central role in the maintenance of immunological homeostasis and host defence in the respiratory tract (Holt *et al.*,

Fig. 6. Determination of endosomal acidification in uninfected *cftr*^{+/+} and *cftr*^{-/-} macrophages. Double labelling of endosomes was conducted by incubating macrophages with 250 µg TMR-dextran ml⁻¹ and 250 µg FITC-dextran ml⁻¹ for 2 h or overnight, followed by 2 h post-incubation in dextran-free medium. Concanamycin (250 nM) and CFTR inhibitor (10 µM) were added 20 min before dextran labelling and were kept throughout the experiment. Confocal images were captured with a 510 LSM confocal microscope with a ×40 objective and fluorescence ratios were analysed using LSM Image Examiner software. (a) Dextran fluorescence in LPS-activated macrophages. Confocal images were captured and fluorescence ratios analysed using a 510 LSM confocal microscope and ×40 objective. Bar, 40 µm. (b) Fluorescence ratios (FITC/TMR) of endosomal compartments in resting macrophages not treated (white bars) and treated (black bars) with concanamycin. CFTRinh-172 denotes CFTR_{inh}-172-treated RAW 264.7 macrophages. The values represent the mean and SD of three experiments involving at least 25 randomly picked macrophages per experiment.

2008). Therefore, although the virulence factors responsible for the persistence of *B. cenocepacia* and the bacterial properties associated with tropism for the airways of CF and other immunocompromised patients are still not fully understood, the ability of these bacteria to survive intracellularly may play a role in pathogenesis (Chiu *et al.*, 2001; Valvano *et al.*, 2005, 2006). The purpose of the present study was to determine whether macrophages carrying a mutated *cftr* gene or a functionally defective CFTR protein have any alterations in their ability to process *B. cenocepacia*. Our results demonstrate that in

CFTR-defective macrophages, the BcCVs exhibit a pronounced delay in phagolysosomal fusion, which is associated with a reduced acidification of the lumen. We also show that this process is specific for live *B. cenocepacia*, since heat-inactivated bacteria are trafficked normally to the lysosomal compartment upon phagocytosis. Furthermore, the non-CF pathogen *S. enterica* does not affect the acquisition of the phagolysosomal marker LAMP-1 in CFTR-defective cells. Di *et al.* (2006) have recently demonstrated that lysosomal compartments within *cftr*^{-/-} alveolar macrophages fail to acidify, leading

to a bactericidal defect, suggesting that CFTR is involved in the pH regulation of the phagocytic vacuole. Our results from ratiometric fluorescent intravacuolar pH measurements do not support the notion that CFTR plays a direct role in the acidification of the phagocytic vacuole, as proposed by Di *et al.* (2006), as we did not find a constitutive acidification defect in our *cfr*^{-/-} macrophages. This is in agreement with a recent detailed analysis using fluorescence ratio imaging to measure the endosomal pH against an internal standard, which convincingly demonstrated that phagolysosomal acidification in macrophages is CFTR-independent (Haggie & Verkman, 2007). It has been reported that in human neutrophils CFTR channel dysfunction affects neutrophil chlorination of phagocytized bacteria (Painter *et al.*, 2006, 2008), raising the possibility that CFTR contributes to bacterial clearance rather than phagosomal acidification. Our experiments with *B. cenocepacia*-infected CFTR-defective macrophages showing an extended delay in the trafficking of BcCVs to lysosomes do indeed support a role for CFTR in the mechanism of clearance of the intracellular infection, as we have shown before that *B. cenocepacia* localized to the lysosome rapidly loses cell envelope integrity (Lamothe *et al.*, 2007). Furthermore, the prolonged maturation arrest of the vacuoles containing *B. cenocepacia* within *cfr*^{-/-} macrophages could explain the persistence of the bacteria within CF patients compared to healthy individuals. Further experiments are currently under way in our laboratory to elucidate the interplay of CFTR and *B. cenocepacia* intracellular survival in macrophages.

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