Delayed association of the NADPH oxidase complex with macrophage vacuoles containing the opportunistic pathogen *Burkholderia cenocepacia*

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Burkholderia cenocepacia causes chronic lung infections in patients suffering from cystic fibrosis and chronic granulomatous disease. We have previously shown that *B. cenocepacia* survives intracellularly in macrophages within a membrane vacuole (BcCV) that delays acidification. Here, we report that after macrophage infection with live *B. cenocepacia* there is a ~6 h delay in the association of NADPH oxidase with BcCVs, while heat-inactivated bacteria are normally trafficked into NADPH oxidase-positive vacuoles. BcCVs in macrophages treated with a functional inhibitor of the cystic fibrosis transmembrane conductance regulator exhibited a further delay in the assembly of the NADPH oxidase complex at the BcCV membrane, but the inhibitor did not affect NADPH oxidase complex assembly onto vacuoles containing heat-inactivated *B. cenocepacia* or live *Escherichia coli*. Macrophages produced less superoxide following *B. cenocepacia* infection as compared to heat-inactivated *B. cenocepacia* and *E. coli* controls. Reduced superoxide production was associated with delayed deposition of cerium perhydroxide precipitates around BcCVs of macrophages infected with live *B. cenocepacia*, as visualized by transmission electron microscopy. Together, our results demonstrate that intracellular *B. cenocepacia* resides in macrophage vacuoles displaying an altered recruitment of the NADPH oxidase complex at the phagosomal membrane. This phenomenon may contribute to preventing the efficient clearance of this opportunistic pathogen from the infected airways of susceptible patients.

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INTRODUCTION

Phagocyte NADPH oxidase plays a crucial role in host defence by professional phagocytes such as neutrophils and macrophages. The NADPH oxidase consists of two membrane-bound (gp91^{phox} and p22^{phox}) and four cytoplasmic subunits (p47^{phox}, p67^{phox}, p40^{phox} and Rac). The enzyme is dormant in resting phagocytes but after activation by soluble stimuli or phagocytosis the complex is assembled and becomes active, generating large quantities of superoxide anions at the plasma or phagosomal membrane (Segal, 2005; Takeya & Sumimoto, 2003). The resulting superoxide is a precursor of more potent microbicidal reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical

and hypochlorous acid (Fang, 2004). Localized production of ROS by the NADPH oxidase complex is spatially regulated and requires the recruitment of the membrane components to the phagosome and the assembly of cytoplasmic regulatory proteins on the phagosomal membrane (Minakami & Sumimoto, 2006).

The integral membrane proteins gp91^{phox} and p22^{phox} form flavocytochrome b₅₅₈, the catalytic centre of the NADPH oxidase (Vignais, 2002). gp91^{phox} belongs to the NADPH oxidase (Nox) family and has six transmembrane helices and two haem moieties, enabling this protein to transport an electron across the membrane. This electron is then donated to an oxygen molecule in the vacuolar lumen, producing the superoxide anion. p22^{phox} has two transmembrane helices and a proline-rich region in the C-terminal cytoplasmic tail. p22^{phox} stabilizes gp91^{phox} at the protein level and acts as an anchor for the soluble regulatory proteins essential for NADPH oxidase function (Minakami & Sumimoto, 2006).

The process of NADPH oxidase assembly has been elucidated in neutrophils, but less is known about the

Abbreviations: AT, 3-amino-1,2,4-triazole; Bcc, *Burkholderia cepacia* complex; BcCV, *Burkholderia cenocepacia*-containing vacuole; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CGD, chronic granulomatous disease; DMEM, Dulbecco's modified Eagle medium; DPI, diphenyleneiodonium chloride; FBS, fetal bovine serum; mRFP1, monomeric red-fluorescent protein 1; NBT, nitro blue tetrazolium; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species.

mechanism of assembly in macrophages. In neutrophils, the disassociation of the cytoplasmic p47^{phox}, p67^{phox} and p40^{phox} ternary complex upon activation results in the phosphorylation and translocation of p47^{phox} to the membrane. p67^{phox} is then recruited via p47^{phox}, with Rac translocating independently of p47^{phox} and p67^{phox} (Bokoch & Diebold, 2002; De Leo *et al.*, 1996; Han *et al.*, 1998; Heyworth *et al.*, 1991). p40^{phox} facilitates the membrane translocation of both p47^{phox} and p67^{phox} but not Rac (Kuribayashi *et al.*, 2002; Ueyama *et al.*, 2007). The NADPH oxidase only produces superoxide after all subunits are correctly translocated to the membrane.

The vital role of NADPH oxidase in innate immunity is clearly illustrated in chronic granulomatous disease (CGD), a rare genetic disorder caused by mutations in several genes encoding subunits of the NADPH oxidase and a subsequent negligible production of vacuolar ROS (Vignais, 2002). CGD patients suffer from recurrent bacterial and fungal infections, which begin early in life, are difficult to treat, and often prove fatal (Dinauer & Orkin, 1992). Many pathogens have evolved various strategies for NADPH oxidase inhibition and avoidance of superoxide-mediated damage. For example, the *Salmonella enterica* pathogenicity island 2 (SPI-2) encodes type III-secreted effector proteins that when translocated into host cells result in mislocalization of NADPH oxidase (Gallois *et al.*, 2001; Vazquez-Torres *et al.*, 2000; Vazquez-Torres & Fang, 2001; Waterman & Holden, 2003). *Anaplasma phagocytophilum* uses a different strategy to suppress the respiratory burst in neutrophils by preventing fusion of bacteria-containing vacuoles with secretory vesicles and specific granules trafficking flavocytochrome b₅₅₈ (Mott *et al.*, 2002). *A. phagocytophilum* can also modify gp91^{phox} promoter activity (Carlyon & Fikrig, 2006). The uptake of *Leishmania donovani* amastigotes by murine macrophages occurs without the phosphorylation of p47^{phox}, abrogating the recruitment of p47^{phox} and p67^{phox} and superoxide production. The amastigotes also disrupt phagosomal lipid raft integrity, potentially interfering with NADPH oxidase assembly (Lodge & Descoteaux, 2006; Lodge *et al.*, 2006). *Helicobacter pylori* also disrupts NADPH oxidase targeting in neutrophils, resulting in the release of superoxide anions into the extracellular milieu, preventing their accumulation in the *H. pylori*-containing phagosome (Allen *et al.*, 2005). Pigments from a number of γ -proteobacteria, termed prodigiosins, inhibit the production of superoxide by preventing the assembly of p47^{phox} and Rac with flavocytochrome b₅₅₈ in RAW 264.7 macrophages (Nakashima *et al.*, 2008).

The *Burkholderia cepacia* complex (Bcc) is a group of closely related bacteria found ubiquitously in the environment (Coenye & Vandamme, 2003; Mahenthalingam *et al.*, 2005). Bcc species have emerged as important multi-drug-resistant pathogens, particularly in patients suffering from cystic fibrosis (CF) (Isles *et al.*, 1984) and CGD (Bylund *et al.*, 2005; Guide *et al.*, 2003). *B. cenocepacia* and

B. multivorans account for the majority of infections in CF patients worldwide (Lipuma, 2005; Mahenthalingam *et al.*, 2008). Bcc species can survive in amoebae (Lamothe *et al.*, 2004; Landers *et al.*, 2000; Marolda *et al.*, 1999), human respiratory epithelial cells (Burns *et al.*, 1996; Keig *et al.*, 2002; Sajjan *et al.*, 2006) and macrophages (Lamothe *et al.*, 2007; Martin & Mohr, 2000; Sajjan *et al.*, 2008). *B. cenocepacia* can also delay the maturation of the phagosome, which may facilitate evasion of host cell microbicidal activities (Lamothe *et al.*, 2007).

Evidence suggests that Bcc strains can overcome oxidative damage by macrophages and neutrophils. In previous studies, we demonstrated that intracellular survival in macrophages infected with Bcc isolates occurs despite an oxidative burst (Saini *et al.*, 1999), and requires production of a periplasmic superoxide dismutase (Keith & Valvano, 2007) and a melanin-like pigment (Keith *et al.*, 2007). Others have shown that the exopolysaccharide capsule material produced by mucoid strains of *B. cenocepacia* can interfere with the function of human neutrophils *in vitro* by inhibiting chemotaxis and ROS (Bylund *et al.*, 2005). However, whether intracellular Bcc bacteria affect the formation of an active NADPH complex has not been directly investigated. Since intracellular *B. cenocepacia* can actively modulate the maturation of the BcCV by delaying acidification and recruitment of late endosomal and lysosomal markers (Lamothe *et al.*, 2007) it is conceivable that the formation of an active NADPH complex may also be altered. In this work, we demonstrate that BcCVs exhibit a delayed association with an NADPH oxidase-positive vacuole, and this delay is further exaggerated in macrophages treated with a specific inhibitor of the cystic fibrosis transmembrane conductance regulator (CFTR). Visualization of superoxide-induced electron-dense deposits *in situ* by transmission electron microscopy demonstrated delayed superoxide formation around BcCVs. Together these data reveal that intracellular *B. cenocepacia* interferes with the formation of an active NADPH oxidase complex in macrophages.

METHODS

Reagents and antibodies. Dulbecco's modified Eagle medium (DMEM), RPMI 1640, PBS and fetal bovine serum (FBS) were obtained from Wisent. Polyclonal primary antibodies [goat anti-mouse p40^{phox} (N-20) antibody and rabbit anti-mouse p22^{phox} (FL195)] were purchased from Santa Cruz Biotechnology. The secondary AlexaFluor 488-conjugated chicken anti-goat and AlexaFluor 647-conjugated goat anti-rabbit antibodies were purchased from Invitrogen. Cerium chloride, phorbol 12-myristate 13-acetate (PMA) and CFTR_{inh}-172 were purchased from Sigma Aldrich. CFTR_{inh}-172 was used at a final concentration of 10 μ M.

Bacterial strains, macrophages and culture conditions. *B. cenocepacia* strain J2315 was chosen as a representative strain of the highly transmissible ET-12 clone (Mahenthalingam *et al.*, 2000). *Escherichia coli* DH5 α [F⁻ ϕ 80lacZ Δ M15 endA recA hsdR(r_K⁻ m_K⁻) supE thi gyrA relA Δ (lacZYA-argF)U169] was from our laboratory stocks. Bacteria were grown in Luria-Bertani (LB) broth at 37 °C with

shaking. *B. cenocepacia* carrying the plasmid pJRL1, which expresses the monomeric red-fluorescent protein 1 (mRFP1) (Lamothe *et al.*, 2007), was grown in the presence of 100 µg trimethoprim ml⁻¹. *E. coli* DH5α carrying the plasmid pRedCm4 was grown with 30 µg chloramphenicol ml⁻¹. This plasmid is a derivative of pME6000 (Lefebvre & Valvano, 2002) that contains the gene encoding mRFP1 and also confers chloramphenicol resistance. The murine macrophage-like cell line RAW 264.7 (TIB-71, ATTC, Manassas, VA, USA) was maintained in DMEM with 10% fetal bovine serum and grown at 37 °C in a humidified atmosphere of 5% CO₂.

Macrophage infection assays. Bacteria were washed twice with HEPES-buffered RPMI 1640 and suspended in DMEM plus 10% fetal bovine serum. For heat inactivation, bacteria were incubated at 60 °C for 25 min prior to infection. We have previously determined that non-opsonic phagocytosis of J2315 by RAW 264.7 macrophages at an m.o.i. of 30–50 results in a reasonable proportion of infected macrophages with a sufficient number of intracellular bacteria for microscopic analysis (Lamothe *et al.*, 2007). Therefore, macrophages were seeded onto glass (or plastic for transmission electron microscopy; see below) coverslips in six-well tissue culture plates and bacteria were added at an m.o.i. of 30, centrifuged at 300 g and incubated at 37 °C under 5% CO₂ for the desired time of infection. Infected macrophages were washed three times with HEPES-buffered RPMI 1640 before fixing and immunostaining.

Immunostaining and confocal microscopy. Cells were fixed with 4% (v/v) paraformaldehyde for 30 min at room temperature and incubated with 100 mM glycine in PBS (1×). Cells were then permeabilized with 0.1% (v/v) Triton X-100 and blocked with 5% (w/v) milk powder for 1 h at room temperature. Permeabilized cells were incubated with primary antibodies, followed by secondary antibodies in 5% (w/v) milk for 1 h each at room temperature. Coverslips were mounted on glass slides using fluorescent mounting medium (Dako). Primary antibodies were used at 1:50 dilution and secondary antibodies were used at 1:1000 dilution. Confocal microscopy was performed using a Zeiss LSM 510 laser scanning confocal microscope with an oil-immersion ×100 objective. For triple-fluorescence experiments bacterial cells expressing mRFP1 were pseudocoloured in blue.

Cerium perhydroxide localization by transmission electron microscopy. RAW 264.7 macrophages were seeded onto either glass or Thermanox plastic coverslips in six-well tissue culture plates and bacteria were added at an m.o.i. of 30, centrifuged at 300 g, and incubated at 37 °C under 5% CO₂ for the desired time of infection. Cells were washed briefly at 4 °C in 0.1 M Tris/maleate buffer, pH 7.5, 7% sucrose and pre-incubated with the same buffer containing 1 mM 3-amino-1,2,4-triazole (AT) at 37 °C for 10 min, and then 10 mM AT, 1 mM CeCl₃ and 0.71 mM NADH at 37 °C for 20 min. Cerium-containing solutions were filter-sterilized prior to use to remove any cerium hydroxide precipitate arising from spontaneous oxidation.

Prior to processing for transmission electron microscopy, macrophages were washed briefly in 0.1 M Tris/maleate buffer, pH 7.5, 7% sucrose at 4 °C and fixed directly on the coverslips using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, containing 5% sucrose, at 4 °C for 1 h. Coverslips were washed in 0.1 M sodium cacodylate buffer, pH 6.0/5% sucrose for 1 h at 4 °C, and overnight in 0.1 M sodium cacodylate buffer, pH 7.3/5% sucrose. Macrophages were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3, for 1 h, and coverslips were washed with 0.1 M sodium cacodylate buffer, pH 7.3. This was followed by dehydration through graded alcohols, infiltration overnight in a 50:50 mixture of absolute ethanol/Epon 812 resin, and infiltration in 100% Epon 812 resin for 2.5 h. Samples were then embedded for sectioning in the

plane of the monolayer, and sections were stained with 2% uranyl acetate and lead citrate and visualized using a Philips 410 transmission electron microscope operating at 60 kV.

Quantification of intracellular superoxide. Intracellular superoxide formation was measured by determining spectrophotometrically the reduction of nitro blue tetrazolium (NBT), which results in insoluble blue deposits inside macrophages (Rook *et al.*, 1985). RAW 264.7 macrophages (2.5 × 10⁵ per well) were seeded into 24-well plates and incubated for 16 h in DMEM + 10% FBS. The medium was aspirated and the cells washed twice with RPMI. NBT at a concentration of 1.25 mg ml⁻¹ was freshly prepared and pre-warmed to 37 °C prior to use. From this solution, 300 µl was added to each well, bacteria were added at an m.o.i. of 30 in DMEM + 10% FBS, and the final volume was brought to 1 ml with DMEM + 10% FBS. As a positive control, 2 µg PMA ml⁻¹ per well was added to uninfected macrophages. Infections were allowed to proceed for the desired time, at which point the supernatant was aspirated and the cells were washed twice with 1 ml RPMI, followed by two washes with 1 ml methanol. The wells were then air-dried and the reduced NBT deposits solubilized by adding 240 µl 2 M KOH per well followed by 280 µl DMSO per well. The A₅₇₀ of the resulting blue solution was determined using a spectrophotometer. Background values were determined by incubation of uninfected RAW 264.7 cells. A standard curve was established by measuring the A₅₇₀ of serial dilutions of a 1 mg NBT ml⁻¹ solution (in 1.2:1.4 2 M KOH/DMSO). The NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) (O'Donnell *et al.*, 1993) was used to determine that NBT reduction was due strictly to the metabolic products of the respiratory burst of the macrophages. All samples were repeated with DPI added to a final concentration of 10 µM at the time of infection; these values were then subtracted from the corresponding well minus the DPI, giving the amount of NADPH oxidase-dependent NBT reduction.

Statistical analyses. Statistical analyses were done using GraphPad Prism version 4.03 (GraphPad Software) by two-way ANOVA and Students' *t*-test, as appropriate.

RESULTS

RAW 264.7 macrophages express the p22^{phox} and p40^{phox} subunits of the NADPH oxidase and become activated by exposure to PMA

We initially evaluated the subcellular localization of the membrane-bound p22^{phox} and cytoplasmic p40^{phox} NADPH oxidase subunits in resting RAW 264.7 macrophages by confocal fluorescence microscopy. Analysis of the distribution of these proteins in macrophage cells demonstrated that p40^{phox} was diffusely localized throughout the cytoplasm while p22^{phox} exhibited punctate staining in the cytoplasm and also localization on the plasma membrane (Fig. 1a). Only sporadically, the subunits showed partial colocalization as shown in the merged images (Fig. 1a, arrows), but for the most part they were clearly found spatially segregated within the cell. Stimulation of the macrophages with PMA results in the activation of protein kinase C, which in turn phosphorylates the cytoplasmic p47^{phox} subunit of the NADPH oxidase complex, causing the complex to assemble and generate superoxide (Nauseef *et al.*, 1991). Stimulation of RAW 264.7 macrophages with 2 µg PMA ml⁻¹ over a

Fig. 1. Distribution of p22^{phox} and p40^{phox} NADPH oxidase subunits in RAW 264.7 macrophages. (a) p22^{phox} and p40^{phox} distribution in resting macrophages. (b) p22^{phox} and p40^{phox} distribution in macrophages after stimulation with 2 µg PMA ml⁻¹ for 15 min. Immunolabelling was performed using goat anti-p40^{phox} and rabbit anti-p22^{phox} primary antibodies. Chicken anti-goat conjugated to AlexaFluor 488 (green fluorescence) and goat anti-rabbit conjugated to AlexaFluor 647 (far red fluorescence) secondary antibodies. Images were captured with an LSM 510 Meta/ConfoCor2 confocal microscope and a ×100 objective. Arrows indicate regions of colocalization in resting macrophages. Bars, 10 µm.

time-course from 0 to 60 min demonstrated that at 15 min virtually 100 % of the macrophage cell population exhibited a complete colocalization of p22^{phox} and p40^{phox} subunits (Fig. 1b). Therefore, these experiments demonstrate that colocalization of p22^{phox} and p40^{phox} can be used as a readout for the assembly of the NADPH complex, and reveal that the NADPH oxidase in RAW 264.7 macrophages can rapidly assemble upon PMA stimulation.

BcCVs of macrophages infected with viable *B. cenocepacia* delay the assembly of the NADPH oxidase on the phagosomal membrane

RAW 264.7 macrophages were infected at an m.o.i. of 30 with *B. cenocepacia* strain J2315 carrying pJRL1, which constitutively expresses the monomeric red-fluorescent protein mRFP1. At 2, 4 and 6 h post-infection, macrophages were rinsed numerous times with prewarmed PBS to remove extracellular bacteria, fixed, and immunostained using anti-p22^{phox} and anti-p40^{phox} antibodies. Extensive rinsing with PBS was preferred over antibiotic treatment since in our experience it is difficult to rely on antibiotics for the removal of extracellular *B. cenocepacia* (Lamothe *et al.*, 2004, 2007; Lamothe & Valvano, 2008; Marolda *et al.*, 1999; Saini *et al.*, 1999), and this method eliminated the majority of extracellular bacteria as visualized microscopically (data not shown). The distribution of p22^{phox} and p40^{phox} subunits over the time-course of infection was expressed as a percentage of the total number of phagosomes that stained positively for p22^{phox} and p40^{phox} subunits (mean ± SD of four separate experiments). At 2 h post-infection, *B. cenocepacia* J2315 was present in BcCVs, and although both p22^{phox} and p40^{phox} were recruited to the general area of the BcCVs, no BcCVs had a characteristic ring of colocalization of either subunit (Fig. 2a). At 4 h post-infection, 24 ± 7 % of BcCVs exhibited a

distinct staining pattern, with both p22^{phox} and p40^{phox} colocalizing on the membrane of the BcCV, forming a ring (Fig. 2b). By 6 h post-infection both p22^{phox} and p40^{phox} subunits were colocalized on the membrane of the majority of BcCVs, as evidenced by rings surrounding internalized bacteria in 82 ± 9 % of BcCVs (Fig. 2c). These results suggest that infection of RAW 264.7 macrophages with viable *B. cenocepacia* J2315 leads to a delayed assembly of the NADPH oxidase complex.

The distribution of p22^{phox} and p40^{phox} was also analysed upon infection of RAW 264.7 macrophages with heat-inactivated *B. cenocepacia*. These experiments were done at 2 h post-infection since at later time points the intracellular bacteria were completely destroyed. BcCVs containing heat-inactivated *B. cenocepacia* were positive for p22^{phox} and p40^{phox}, with both subunits forming complete rings of colocalization around the phagosomes (Fig. 2d). To confirm that the delay in NADPH oxidase assembly was specifically dependent on live *B. cenocepacia*, RAW 264.7 macrophages were infected with *E. coli* DH5α(pRedCm4), which constitutively expresses mRFP1. At 1 h post-infection, the p22^{phox} and p40^{phox} colocalized to virtually all phagosomes containing *E. coli* (Fig. 2e). Also, like heat-inactivated *B. cenocepacia*, *E. coli* cells displayed morphologies suggesting a compromised membrane, including the release of cytoplasmic mRFP1 into the vacuolar lumen, and at later time points, bacteria were destroyed by the macrophages (data not shown). Together, these results suggest that live *B. cenocepacia* is required for the delayed assembly or recruitment of the NADPH phagocyte oxidase on the BcCV membrane.

The absence of intracellular *B. cenocepacia* replication compounded with the failure to kill extracellular bacteria because of their extraordinary resistance to cell-membrane-impermeable antibiotics prevents us from obtaining viable

Fig. 2. Distribution of p22^{phox} and p40^{phox} NADPH oxidase subunits in infected RAW 264.7 macrophages at various time points. (a, b, c) Macrophages infected with viable *B. cenocepacia* J2315(pJRL1) were examined at 2 h (a), 4 h (b) and 6 h (c) post-infection. (d) Macrophages infected with heat-inactivated *B. cenocepacia* J2315 at 2 h post-infection. (e) Macrophages infected with viable *E. coli* DH5 α (pRedCm4) at 1 h post-infection. Immunolabelling and image capture were performed as described in Fig. 1. Bacterial cells were pseudocoloured in blue. Bars, 10 μ m.

counts in macrophage infection experiments (Lamothe *et al.*, 2007; Saini *et al.*, 1999). However, we can conclude that intracellular *B. cenocepacia* remained viable during the course of the infection as demonstrated by the retention of mRFP1 within the bacterial cytoplasm. We have previously demonstrated that intracellular *B. cenocepacia* reaching the lysosomes rapidly lose cell envelope integrity, resulting in the dispersal of the fluorescent protein throughout the phagosomal lumen, which serves as an indication of bacterial cell disruption (Lamothe *et al.*, 2007). This is particularly obvious with heat-killed bacteria, which retain the fluorescence if they are kept in buffer but leak fluorescence into the phagosomal lumen after phagocytosis (Lamothe *et al.*, 2004, 2007). Therefore, retention of mRFP1 within the bacterial cytoplasm in more than 80 % of the vacuoles after infection with live *B. cenocepacia* (data not shown) provides good evidence that the bacterial cell envelope remains intact, and is consistent with previous observations in other studies (Lamothe *et al.*, 2007; Maloney & Valvano, 2006; Saldías *et al.*, 2008).

Inhibition of CFTR function increases the delay in NADPH oxidase assembly

CF patients carry mutations in a gene encoding a cAMP-regulated chloride channel called the CFTR (Cheng *et al.*, 1990). We have recently observed that after infection with *B. cenocepacia*, the BcCVs in CFTR-defective macrophages or macrophages pre-treated with a CFTR functional inhibitor exhibit a more extended delay in acidification and phagolysosomal fusion than that usually observed in wild-type macrophages (Lamothe & Valvano, 2008). The CFTR-associated phagosomal maturation defect is only detected upon infection of macrophages with live *B. cenocepacia*, and is absent in macrophages exposed to heat-inactivated bacteria or macrophages infected with a non-CF pathogen such as *Salmonella enterica* (Lamothe & Valvano, 2008). To examine whether the absence of CFTR function has any effect on the assembly of the NADPH oxidase complex upon infection with *B. cenocepacia*, RAW 264.7 macrophages were incubated for 30 min prior to infection with the CFTR functional inhibitor molecule CFTR_{inh}-172 (Ma *et al.*, 2002). The distribution of p22^{phox} and p40^{phox} was assessed by immunostaining and confocal microscopy as described above. At 2 h post-infection p22^{phox} and p40^{phox} were spatially segregated in the macrophage, with a distribution similar to that observed for resting macrophages in Fig. 1(a) (data not shown), while at 4 h post-infection there was partial colocalization of the p22^{phox} and p40^{phox} subunits (Fig. 3a). These areas of colocalization were in the vicinity of the BcCVs and did not form distinctive rings on the BcCV membrane as described at the 4 h time point in the absence of the CFTR inhibitor (Fig. 2b). At 6 h post-infection both p22^{phox} and p40^{phox} were found to colocalize, particularly on the plasma membrane of the macrophage, but not specifically on the membrane of the BcCV (Fig. 3b). Some colocalization of the subunits was observed in the vicinity of the BcCVs, but

the distinctive rings of colocalization that were observed on the membrane of the BcCV at 6 h post-infection with no inhibitor (Fig. 2c) were seen in only a small percentage of BcCVs. The kinetics of the association of BcCVs with p22^{phox} and p40^{phox} rings were assessed over time, with at least 150 BcCVs counted from 21 different fields of view per time point (Fig. 4). At 2 h post-infection, with and without the CFTR inhibitor, no rings of NADPH oxidase colocalization were observed on the BcCV membrane. At 4 h post-infection, rings of colocalization were observed in 23 ± 6 % of BcCVs; this number was reduced almost 16-fold to 1.5 ± 0.7 % when macrophages were pre-incubated with the CFTR inhibitor. At 6 h post-infection, rings of colocalization were observed in 82 ± 9 % of BcCVs, but this number was reduced almost 8-fold to 10 ± 8 % when macrophages were pre-incubated with the CFTR inhibitor. Since CFTR_{inh}-172 specifically reduces CFTR function (Ma *et al.*, 2002), these results suggest that the loss of CFTR function in RAW 264.7 macrophages further increases the delay in assembly of the NADPH oxidase complex on the plasma membrane of the BcCV. To investigate if this delay was specific to the inhibition of CFTR, or due to CFTR inhibition in combination with *B. cenocepacia* infection, these experiments were repeated with *E. coli* and heat-inactivated *B. cenocepacia*. At 2 h post-infection with heat-inactivated *B. cenocepacia* the addition of the CFTR inhibitor did not delay the assembly of the NADPH oxidase on the BcCV membrane, and complete rings of colocalization were observed (data not shown). Pre-incubation with the CFTR inhibitor followed by a 1 h infection with *E. coli* did not delay the assembly of the NADPH oxidase on the phagosomal membrane (Fig. 3c). Also, treatment with the CFTR_{inh}-172 inhibitor did not prevent degradation of *E. coli* within the phagosome, as previously observed for the infection in the absence of inhibitor. We conclude from these results that the delayed NADPH oxidase assembly phenotype observed upon infection with live *B. cenocepacia* is enhanced in the presence of the CFTR functional inhibitor.

Intracellular superoxide production during *B. cenocepacia* infection of RAW 264.7 macrophages and visualization of NADPH oxidase activity in relation to the BcCV

To determine the effect of *B. cenocepacia* infection on the formation of superoxide by macrophages, we measured superoxide release using the NADPH-dependent reduction of NBT. Macrophages were subjected to a time-course of *B. cenocepacia* infection and the amount of DPI-inhibitable NBT reduction was measured as described in Methods. Using DPI, an inhibitor of the NADPH oxidase, allowed us to determine NBT reduction levels caused strictly by the metabolic products of the macrophages' respiratory burst. At 2 h post-infection 18 ± 4 µg DPI-inhibitable NBT reduction ml⁻¹ was observed; this increased to 38 ± 1 µg ml⁻¹ at 4 h and to 56 ± 3 µg ml⁻¹ at 6 h. Infection for 2 h with live or heat-inactivated *E. coli* resulted in 30 ± 4 µg

Fig. 3. Effect of CFTR inhibition on the distribution of p22^{phox} and p40^{phox} NADPH oxidase subunits in RAW 264.7 macrophages treated with CFTR_{inh}-172. (a) Distribution at 4 h post-infection with *B. cenocepacia* J2315. (b) Distribution at 6 h post-infection with *B. cenocepacia* J2315. (c) Distribution at 1 h post-infection with *E. coli* DH5 α . Immunolabelling and image capture were performed as described in Fig. 1. Bacterial cells were pseudocoloured in blue. Bars, 10 μ m.

ml⁻¹ and 55 ± 7 μ g ml⁻¹ of DPI-inhibitable NBT reduction, respectively. Infection with heat-inactivated *B. cenocepacia* for 2 h resulted in 25 ± 3 μ g ml⁻¹ of DPI-inhibitable NBT reduction. These results demonstrate that the amount of superoxide produced by RAW 264.7 macrophages infected with live *B. cenocepacia* is reduced at similar time points when compared to *E. coli* and heat-inactivated controls (Fig. 5).

To determine the *in situ* formation of superoxide in the vicinity of the BcCVs, ultrastructural studies were performed on infected RAW 264.7 macrophages that were treated with cerium chloride (Fig. 6). Upon exposure to hydrogen peroxide, cerium chloride is converted to an electron-dense cerium perhydroxide precipitate (Telek *et al.*, 1999). Transmission electron microscopy revealed that at 2 h post-infection BcCVs did not show detectable deposits of cerium perhydroxide (Fig. 6b, c). Small deposits of cerium perhydroxide were present on the plasma

membrane of the macrophage (Fig. 6a). At 4 h post-infection, electron-dense precipitates were observed on the membrane of some BcCVs, forming punctate patterns, but only a minority of BcCVs had complete rings of precipitate on the membrane (Fig. 6d) and some BcCVs had no precipitate at all (Fig. 6e, f). In contrast, at 6 h post-infection most BcCVs had rings of precipitate on their membranes (Fig. 6g–i). These results confirm that the NADPH oxidase is assembled on the membrane of BcCVs and is active by 6 h post-infection. As controls, macrophages infected with *E. coli* and heat-inactivated *B. cenocepacia* were also examined by transmission electron microscopy. Rings of precipitate were seen on the membranes of vacuoles containing *E. coli* (Fig. 6j, k) and heat-inactivated *B. cenocepacia* (Fig. 6l) at 1 h and 2 h post-infection, respectively. Therefore, the histochemical localization of superoxide production parallels the observed colocalization kinetics for p22^{phox} and p40^{phox} NADPH oxidase subunits, supporting the notion that

Fig. 4. Kinetics of association of BcCVs with p22^{phox} and p40^{phox} NADPH oxidase subunits with and without pre-incubation with CFTR_{inh}-172. Values represent the mean $\pm$ SD of three independent experiments in which at least 150 BcCVs from 21 fields of view were examined at $\times 100$.

BcCVs arising from infection of macrophages with viable *B. cenocepacia* delay the assembly of a functional NADPH oxidase complex.

DISCUSSION

Chronic lung infection progressively compromises lung function in CF patients, ultimately leading to the death of the patient. Macrophages play a central role in immunological homeostasis and host defence of the respiratory

Fig. 5. Quantification of intracellular superoxide production in RAW 264.7 macrophages based on DPI-inhibitable NBT reduction as described in Methods. Macrophages were treated with PMA for 15 min, or exposed to *E. coli*, viable *B. cenocepacia* J2315 or heat-inactivated (HK) *B. cenocepacia* J2315 for the indicated times. Values represent the mean $\pm$ SD of three independent experiments.

tract (Holt *et al.*, 2008). The virulence factors responsible for the persistence of *B. cenocepacia* and other Bcc bacteria, as well as the specific bacterial properties associated with tropism for the airways of CF and other immunocompromised patients, are still not fully understood. Intracellular survival of Bcc bacteria within macrophages and epithelial cells may contribute to bacterial persistence within the lung and airways of CF patients, and sustained tissue inflammation and subsequent tissue destruction (Chiu *et al.*, 2001; Valvano *et al.*, 2005; Valvano, 2006).

In this study, we demonstrate that *B. cenocepacia* is initially internalized into a BcCV that does not colocalize with either p22^{phox} or p40^{phox} for at least 2 h post-infection, followed by a progressive increase in the association of both NADPH oxidase subunits until ~ 6 h, when association reaches more than 80%. This delay in assembly of NADPH oxidase subunits on the membrane of the phagosome was specific to live *B. cenocepacia*, as both heat-inactivated *B. cenocepacia* and live *E. coli* are present in a phagosome that completely colocalizes with p22^{phox} and p40^{phox} NADPH oxidase subunits at 2 and 1 h post-infection, respectively. The confocal microscopy experiments did not provide information about the activity of the NADPH oxidase once it was assembled on the BcCV. Thus, we used the NADPH-dependent reduction of NBT as a quantitative measure of total superoxide production together with ultrastructural histochemistry studies with cerium chloride to show that anion superoxide is formed and progressively accumulates on the BcCV membrane, in parallel with the observed kinetics of NADPH oxidase subunit assembly.

The specific mechanism utilized by *B. cenocepacia* to delay the recruitment/assembly of a functional NADPH oxidase complex on its phagosomal membrane is unknown. In other bacteria such as *Salmonella enterica* type III secreted effectors mediate the exclusion of NADPH oxidase components from the *Salmonella*-containing vacuole (Gallois *et al.*, 2001; Vazquez-Torres *et al.*, 2000). The effectors responsible for this exclusion are believed to act through tumour necrosis factor receptor p55 (Vazquez-Torres & Fang, 2001). We have previously demonstrated that a polar mutation introduced in the first gene of the *B. cenocepacia* J2315 type III secretion gene cluster does not abolish the BcCV maturation delay (Lamothe *et al.*, 2007), arguing that type III secreted effectors are likely not involved in the delay of NADPH oxidase assembly. Recently, it has been reported that type IV secretion is required for the intracellular survival of *B. cenocepacia* K56-2 (Sajjan *et al.*, 2008), a strain clonally related to J2315 (Mahenthalingam *et al.*, 2000), suggesting the possibility that an unidentified type IV secreted effector may be involved in the BcCV maturation delay. We have also observed that *B. cenocepacia* possesses a type VI secretory system that causes disruptions in the actin cytoskeleton (Aubert *et al.*, 2008). There is evidence that components of the NADPH complex, including p40^{phox}, associate with the actin cytoskeleton (Chen *et al.*, 2007). Therefore, we are

Fig. 6. Visualization of NADPH oxidase activity in *B. cenocepacia*-infected RAW 264.7 macrophages treated with cerium chloride as described in Methods. Deposits of electron-dense cerium perhydroxide indicate superoxide formation, suggesting NADPH oxidase activity. Arrows point to the membrane of bacteria-containing vacuoles. Macrophages infected with viable *B. cenocepacia* were examined at 2 h (a–c), 4 h (d–f), and 6 h (g–i) post-infection, respectively. Macrophages infected with *E. coli* were examined at 1 h post-infection (j–k). Macrophages exposed to heat-inactivated *B. cenocepacia* were examined at 2 h post-infection. Scale bars are as follows: (a), (d) and (h), 1 μ m; (b), (c), (i), (j), (k) and (l), 0.1 μ m; (e), (f) and (g), 0.5 μ m.

currently investigating whether type VI and perhaps also type IV secreted bacterial effectors could be involved in the NADPH oxidase assembly delay. Other researchers have noticed that *B. cenocepacia* can cause cell death of neutrophils from patients with CGD (Bylund *et al.*, 2005) and also human dendritic cells (Macdonald & Speert, 2008). Therefore, an alternative possibility to explain our results is that the intracellular infection may induce cell death in macrophages and the mislocalization of the NADPH oxidase may be associated with this process. However, macrophages do not show any evidence of loss of viability as determined by trypan blue exclusion assays and lactic acid dehydrogenase release during the 6 h time-

course of our experiments (L. S. Saini & M. A. Valvano, unpublished).

Little is known regarding whether there is any link between the CFTR defect and the increased susceptibility of CF patients to opportunistic pathogens such as *B. cenocepacia*. Recently, it was suggested that phagolysosomes in alveolar macrophages have a CFTR-dependent constitutive defect in acidification that could lead to impaired bactericidal activity (Di *et al.*, 2006). In contrast, a detailed analysis using fluorescence ratio imaging to measure the endosomal pH against an internal standard convincingly demonstrated that phagolysosomal acidification in macrophages is

CFTR-independent (Haggie & Verkman, 2007). It has also been demonstrated that inhibition of CFTR activity is responsible for the onset of the inflammatory cascade in the CF lung (Perez *et al.*, 2007). Our independent studies using a pair of *cfr*-null and *cfr*^{+/+} murine macrophages support the notion that CFTR is not directly involved in phagosomal acidification (Lamothe & Valvano, 2008). However, we have also observed that in *cfr*-null macrophages as well as in normal macrophages treated with the CFTR_{inh}-172 the BcCVs exhibit an even more prolonged maturation delay that is only manifested upon infection with live *B. cenocepacia* (Lamothe & Valvano, 2008). Here, we examined whether the status of CFTR affects the association of the NADPH oxidase with BcCVs. The results clearly show that in macrophages treated with CFTR_{inh}-172 there is a further delay in NADPH oxidase assembly on the membrane of the BcCV. The prolonged delay in colocalization of the NADPH oxidase at the phagosomal membrane in the presence of the CFTR inhibitor did not occur when macrophages were infected with either heat-inactivated *B. cenocepacia* or *E. coli* controls, suggesting a specific interaction between CFTR and a *B. cenocepacia*-specific component.

In conclusion, the experiments reported here add new knowledge about the characteristics of the intracellular infection of macrophages by *B. cenocepacia* by showing that in addition to reduced acidification and delayed fusion with late phagolysosomal markers (Lamothe *et al.*, 2007), BcCVs exhibit a delay in the normal assembly of a functional NADPH oxidase complex on their membrane. Our experiments also support a role for CFTR in the clearance of the intracellular infection by *B. cenocepacia*, which might help explain why CF patients are preferentially infected by *Burkholderia* species over a myriad of other antibiotic multi-resistant environmental opportunistic pathogens.

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