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**Highlights**

- CFTR impairment decreases the phagocyte oxidative defense by limiting ROS production
- CFTR is required for neutrophil recruitment toward forming granulomas
- Granuloma breakdown leads to extracellular mycobacterial expansion
- Dysfunction of CFTR results in hypersusceptibility to *M. abscessus* infection

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**In Brief**

Bernut et al. investigate the mechanism by which cystic fibrosis patients are vulnerable to *Mycobacterium abscessus* infection. Using zebrafish, they show that dysfunction of CFTR reduces both macrophage bactericidal activity and neutrophil recruitment to the forming protective granulomas. Together, this leads to hypersusceptibility to *M. abscessus* infection and larval death.
CFTR Protects against *Mycobacterium abscessus* Infection by Fine-Tuning Host Oxidative Defenses

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SUMMARY

Infection by rapidly growing *Mycobacterium abscessus* is increasingly prevalent in cystic fibrosis (CF), a genetic disease caused by a defective CF transmembrane conductance regulator (CFTR). However, the potential link between a dysfunctional CFTR and vulnerability to *M. abscessus* infection remains unknown. Herein, we exploit a CFTR-depleted zebrafish model, recapitulating CF immuno-pathogenesis, to study the contribution of CFTR in innate immunity against *M. abscessus* infection. Loss of CFTR increases susceptibility to infection through impaired NADPH oxidase-dependent restriction of intracellular growth and reduced neutrophil chemotaxis, which together compromise granuloma formation and integrity. As a consequence, extracellular multiplication of *M. abscessus* expands rapidly, inducing abscess formation and causing lethal infections. Because these phenotypes are not observed with other mycobacteria, our findings highlight the crucial and specific role of CFTR in the immune control of *M. abscessus* by mounting effective oxidative responses.

INTRODUCTION

Cystic fibrosis (CF) is a lethal genetic disorder caused by deleterious mutations in the CF transmembrane conductance regulator (CFTR) protein (Gadsby et al., 2006), resulting in compromised mucociliary clearance, chronic bacterial infections, and subsequent progressive inflammatory lung damage (Donaldson and Boucher, 2003). CF-related lung infections are associated with a specific spectrum of colonizing microorganisms: highly prevalent bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Lyczak et al., 2002) and also emerging bacterial pathogens such as nontuberculous mycobacteria (NTM) (Olivier et al., 2003; Roux et al., 2009). Among the rapidly growing NTM, the *Mycobacterium abscessus* complex (MABSC) represents the most common species found in CF airways (Floto et al., 2016) and is emerging as a major CF pathogen, in part because of indirect person-to-person transmission (Bryant et al., 2013), and progressing into severe pneumonia and accelerated inflammatory lung damage (Esther et al., 2010). Their presence is also a relative contraindication to lung transplantation (Orens et al., 2006). In particular, pulmonary infections with the multidrug-resistant *M. abscessus* subspecies (subsp.) *abscessus* (Mabs) (Nessar et al., 2012) are extremely challenging to treat, requiring aggressive and extended therapies with a high rate of therapeutic failure (DaCosta et al., 2017). To date, Mabs is widely considered to be the most significant rapidly growing NTM in CF, with a worldwide prevalence rate of 5%–20% (Floto et al., 2016).

Mabs exhibits two distinct morphotypes, relying on the presence or absence of surface-associated glycopeptidolipids (GPLs): a smooth (S) high-GPL producer variant and a rough (R) low-GPL producer variant (Howard et al., 2006). Both morphotypes are recovered from the CF airways during infection, but case reports indicate that the R form correlates with exacerbations of pulmonary disease and rapid decline of lung function in the patients (Jonsson et al., 2007; Catherinot et al., 2009). Nevertheless, the specific vulnerability of the CF population to Mabs, the potential link with CFTR dysfunction, and how these mycobacteria contribute to progression of lung disease remain unknown.

Although it is assumed that susceptibility to infections in CF results from defective mucociliary activity, CFTR dysfunction may also alter the inflammatory potential of innate immune cells, contributing to the infectious pathology in this disease. Various hypotheses have attempted to explain the impairment of innate defenses in CF, although these await definitive proof. Numerous mammalian models (Lavelle et al., 2016) have been generated to investigate the role of CFTR dysfunction in innate immunity and hypersusceptibility to infections. However, these models are not suited for direct, real-time imaging of the early processes leading to disease development. Thus, new animal models that approximate the human altered immune phenotype and allow direct visualization of host-pathogen interactions would provide
much needed tools to establish in vivo how CFTR regulates innate immunity and controls Mabs infection.

Zebrafish (ZF) larval innate immunity is homologous to that of human (Renshaw and Trede, 2012), and their optical transparency allows non-invasive, real-time monitoring of infection outcomes and host-pathogen interactions. Thus, ZF innate immune cell behavior and function can be visualized at sub-cellular resolution in the whole living animal, allowing dissecting the innate immune response during infectious diseases (Torraça et al., 2014). ZF led to new insights into the virulence of several CF bacteria, especially P. aeruginosa (Clatworthy et al., 2009), S. aureus (Prajsnar et al., 2008), and Burkholderia cenocepacia (Vergunst et al., 2010), and provide a useful model to study the pathophysiology of human Mabs infection (Bernut et al., 2014, 2016a). Importantly, from a structural perspective, ZF CFTR closely resembles the human protein (Zhang and Chen, 2016; Liu et al., 2017), and several reports suggest functional conservation of CFTR between ZF and human (Navis and Bagnat, 2015; Phennicie et al., 2010), making ZF larvae a clinically relevant biological system. Indeed, several CF phenotypes that mirror human CF disease were reported in CFTR-defective ZF, including pancreatitis (Navis and Bagnat, 2015) and increased susceptibility to P. aeruginosa (Phennicie et al., 2010).

Herein, using CFTR-depleted ZF larvae as an innovative vertebrate model that recapitulates important aspects of the CF immuno-pathogenesis, we elucidated the role of CFTR in regulation of innate immunity to Mabs infections. We also interrogated the effects of CFTR ablation on host immunity, inflammation, and infection independent of the overlapping infection and inflammation associated with the CF-lung microenvironment. Importantly, our findings emphasize that differential CFTR-dependent ROS production allows the host to adjust inflammatory responses by modulating phagocyte bactericidal functions and their lifespan upon infection, which together ensure the maintenance of a protective granulomatous structure to sequester and control Mabs infection.

RESULTS

Loss of CFTR Function Increases the Severity of M. abscessus Infection

To address the role of CFTR in Mabs infection, cftr loss-of-function experiments were carried out in ZF using a specific morpholino-modified oligonucleotide (MO) (Figures S1A and S1B). Although cftr is expressed and localized to the apical membrane or vesicular compartments of cells (Del Porto et al., 2011) (Figure S1C), cftr-MO injection abrogated production of native spliced cftr transcripts (Figures S1A and S1B) and altered cftr expression (Figure S1D). Embryos injected with cftr-MO survived similarly to control-MO injected animals and appeared morphologically similar throughout the observed periods (data not shown). Additionally, to support the knockdown results, we took advantage of the cftr ZF mutant (Navis et al., 2013). Upon intravenous infection, both cftr morphants and cftr mutants displayed hypersusceptibility to R and S Mabs morphotypes, correlating with increased larval mortality (Figure 1A) and higher bacterial burdens, as demonstrated by determination of the fluorescent pixel count (FPC; Figure 1B) and whole-larvae imaging (Figure 1C). The pronounced increase in bacterial loads in CFTR-deficient animals correlates with replicating extracellular bacteria (Figures 1D–1H), translating into larger numbers of larvae with abscesses and with increased number of abscesses per larva in the CNS (Figures 1D and 1E). Mabs abscesses represent a marker of disease severity and uncontrolled infection caused by extracellular replicating mycobacteria that are often associated with cellular debris, tissue destruction, and acute infection in ZF (Bernut et al., 2014). Whereas the S form induces abscesses only rarely in wild-type (WT) fish (Bernut et al., 2014), 30% of Mabs-S-infected larvae exhibited abscesses at 3 days post-infection (dpi) in the absence of CFTR (Figure 1D). Electron microscopy (EM) analysis revealed that Mabs S abscesses in cftr morphant show enhanced replication of extracellular bacilli, promoting rapid bacterial expansion and tissue destruction (Figure 1F), similar to those reported in Mabs R abscesses found in WT fish (Bernut et al., 2014). Moreover, hypersusceptibility to Mabs infection in CF fish is accompanied by increased bacterial cording in Mabs R-infected animals compared with WT larvae (Figures 1G and 1H). Collectively, these results indicate that cftr mutants recapitulate phenotypes induced by cftr-MO, implying that hypersusceptibility to Mabs in cftr morphants is not ascribed to off-target effects but to the direct consequences of cftr loss, thus validating the use of cftr-MO to further investigate the role of CFTR in innate immunity to Mabs.

Other MABSC subsp. or the rapid-growing NTM Mycobacterium chelonae, which is closely related to Mabs, can be isolated from CF expectorated sputum (Harris and Kenna, 2014). Infection of cftr morphants with M. abscessus subsp. massiliense (Figure S2A), M. abscessus subsp. bolletii (Figure S2B), or M. chelonae (Figure S2C) led to increased susceptibility to infections and larval killing, similarly to Mabs-infected CFTR-defective animals (Figure 1). In contrast, neither the non-pathogenic Mycobacterium smegmatis (Figure S2D) nor Mycobacterium marinum, one of the strict pathogenic NTM and closely related to Mycobacterium tuberculosis (Figure S2E), induced increased larval killing in the absence of CFTR. These results indicate that the susceptibility to mycobacterial infections in CFTR-deficient embryos is specific and restricted to particular NTM species, such as those belonging to the M. chelonae complex (MCC), comprising MABSC and M. chelonae, and emphasize the protective role of CFTR in response to MCC infection by restricting bacterial pathogenesis and extracellular multiplication.

CFTR Deficiency Compromises M. abscessus Granuloma Maintenance by Permitting Rapid Mycobacterial Extracellular Expansion

Having previously demonstrated the importance of Mabs-induced granuloma formation and maintenance to prevent extracellular bacterial expansion and ensure the host defense (Bernut et al., 2014, 2016a), the increased mortality and extracellular mycobacterial growth in Mabs-infected CFTR-depleted animals prompted us to (1) further characterize the granulomatous response to Mabs in the absence of CFTR and (2) ask if CFTR influences the course of granuloma formation and/or granuloma composition. Mabs-infected WT, cftr mutants, and cftr
morphants were compared and monitored over time for granuloma formation by fluorescent microscopy. In agreement with previous observations, Mabs-granulomatous lesions were found in both the presence and absence of CFTR (Figures 2A–2G) (Tomashefski et al., 1996). However, although nascent granuloma appeared at 2 dpi and expanded in most infected WT
Figure 2. Absence of CFTR Impairs *M. abscessus* Infection-Mediated Granuloma Integrity and Maintenance

(A–D) WT, *cfr* mutants, and *cfr* morphants were i.v. infected with the S variant of *Mabs* expressing tdTomato and monitored using confocal microscopy for granuloma formation, number, and size (n = 35–45; data are plotted as mean ± SEM from three experiments).

(A and B) Kinetics of granuloma formation in whole embryos over 4 days of infection (A) and associated number of granuloma per animal (B).

(legend continued on next page)
embryos at 4 dpi (Figure 2A) (Bernut et al., 2014, 2016a), time-lapse microscopy revealed that the granuloma formation in CFTR-defective larvae remained unchanged, with fewer granulomas observed in about 20%–30% of the animals. No differences in the proportion of S or R granulomas were noticed in the context of CFTR impairment (data not shown). Additionally, confocal imaging showed that early granuloma formation and cellular aggregation events are maintained in the absence of CFTR function (Figure 2C), suggesting that granuloma elaboration proceeds regardless whether CFTR is present or not. Although this difference was not statistically significant, interestingly, at all time points, there was a trend toward increased average size of granuloma lesions in cftr morphants, which appeared more heavily infected than WT granulomas (Figures 2C and 2D) and continued expanding concomitantly with a time-dependent increased bacterial burden. This suggests that dysfunction of CFTR leads to a persistent acceleration in Mabs granuloma growth beyond the initial aggregation event. Next, the late granuloma-like structure in the absence of CFTR was explored by confocal imaging (Figures 2E and 2G) and EM (Figures 2F and 2H). Although the structures characterizing WT granulomas contained organized aggregates of phagocytes (Figure 2E) consisting of infected and uninfected cells surrounding a central necrotic region (Figure 2F), which efficiently control and sequester Mabs, we confirmed that impaired-CFTR granulomas are degenerated (Figures 2G and 2H), supporting the hypothesis that CFTR deficiency compromises granuloma maintenance. Overall, late CF granulomas appear poorly delimited and contain dissociated cellular aggregates (Figures 2G and 2H) with abundant extracellularly replicating bacilli forming abscesses within tissues, presumably responsible for the pronounced increase in phagocyte death observed in the granulomas (Figure 2H). In sharp contrast to WT Mabs granuloma, microscopy revealed the profusion of highly infected phagocytes in CF granulomatous lesions (Figure 2C), suggestive of impaired bacterial killing (Bernut et al., 2014, 2016a).

Together, these results indicate that a CFTR defect triggers the breakdown of Mabs granulomas typified by extracellularly growing bacteria released from dying phagocytes. This implies that CFTR is indispensable for normal granuloma structure and maintenance by controlling the phagocyte bactericidal functions and lifespan and preventing extracellular multiplication.

Dysfunction of CFTR Impairs Killing of M. abscessus in Macrophages

Our previous studies using Mabs-infected ZF highlighted the crucial role of macrophages for Mabs killing and infection control (Bernut et al., 2014, 2016a). In CF pathophysiology, the role of macrophages has been largely overlooked: scant evidence suggests altered macrophage properties in uncontrolled infection in CF lungs (Döring and Gubulis, 2009). To elucidate the cellular basis linking CFTR deficiency with susceptibility to Mabs infection and alteration of the macrophage functions, expression of cftr was knocked down in reporter lines harboring labeled macrophages. We first examined if the lack of CFTR affects chemoattraction of macrophages to the invading bacteria and/or activation of these cells, as potential mechanisms promoting extracellular bacterial growth. Leucocyte mobilization was assessed by injecting fluorescent Mabs into the hind-brain ventricle (HBV), and their phagocytic capacity was monitored after intravenous infection of the bacilli. Deficiency of cftr compromised neither mycobacterial-induced migration to the infected HBV (Figure 3A) nor phagocytosis (Figure 3B) at early time points, suggesting that CFTR is not required for early interactions between Mabs and macrophages. Previous studies reported that dysfunction of CFTR is associated with reduced microbicidal capacities of immune cells (Assani et al., 2017; Di et al., 2006; Duranton et al., 2012). However, recent studies have shown comparable intracellular Mabs growth in murine macrophages carrying the CFTR<sup>F508</sup> mutation and in WT macrophages, suggesting that functional CFTR is not required for the control of Mabs in murine macrophages infected <i>ex vivo</i> (Roux et al., 2016). Nevertheless, our microscopic observations show that CFTR ablation leads to hyperinfected phagocytes in granulomas, presumably relying on altered immune bacterial killing mechanisms (Figure 2C). Thus, to interrogate whether CFTR contributes to the macrophage mycobactericidal capacity <i>in vivo</i>, the number of Mabs in ZF macrophages was evaluated using confocal microscopy. The proportion of slightly infected (< 5 bacilli), moderately infected (5–10 bacilli), or heavily infected (> 10 bacilli) phagocytes was enumerated at 1 dpi. Compared with the control embryos, the cftr morphants infected by both R and S variants displayed a greater percentage of macrophages in the high-burden category (Figure 3C). This is consistent with a reduced bactericidal ability and supports the hypothesis that CFTR controls...

(C) Confocal images showing a representative early Mabs granuloma in 3 dpi larvae. Scale bars, 10 μm. For a similar number of infected phagocytes (asterisk) in a granuloma, the CF granuloma contains a higher bacterial burden than the WT granuloma.

(D) Granuloma volume analysis in whole larvae over 4 days of infection.

(E–H) Control larvae or cftr morphants infected with Mabs S expressing tdTomato observed by confocal microscopy and EM for granuloma at 4 dpi.

(E and G) Representative confocal imaging of a granuloma within the spinal cord of an infected animal showing the development of a compact and organized phagocyte aggregate moderately infected in control fish (E) compared with a cftr morphant harboring a heavily loaded granuloma whose disruption leads to bacterial spread characterized by abscess formation (G). Scale bars, 10 μm.

(F) EM showing a sagittal section through a WT Mabs granuloma. Overview image of granuloma (arrow) into the brain of infected WT (top; scale bar, 50 μm). Representative EM image of a compact and well-organized WT granuloma (bottom and right; processed as stitching of micrographs; scale bars, 2 μm) showing a stable mycobacterial-containing structure with a central necrotic area and a typical dense region of cellular debris and surrounded by numerous infected phagocytes (asterisk) and giant cells (arrow).

(H) EM showing a sagittal section through a Mabs granuloma-like lesion in absence of CFTR. Overview image of granuloma in the spinal cord (arrow) of infected cftr morphant (top; scale bar, 25 μm). Representative EM image of a CFTR-depleted granuloma (bottom and right; processed as stitching of micrographs; scale bar, 2 μm) showing a necrotic structure with replicating bacteria and numerous dead infected phagocytes harboring a typical apoptotic nucleus (asterisk) with chromatin “superaggregation.” Most bacilli stay extracellular.
intracellular growth and killing of Mabs. Because death of Mabs-infected phagocytes releases and propagates free bacilli in the extracellular milieu (Bernut et al., 2014), we examined the extent of macrophage death in infected larvae. Combined confocal observations and quantification of acridine orange (AO)-positive infected macrophages shows that infection in cftr morphants is characterized by the presence of heavily infected phagocytes with an impaired ability to restrict bacterial growth, occurring prior to cell death (Figure 3D). Although the basal levels of dead macrophages were equal between the PBS-injected control embryos and cftr morphants (data not shown), enumeration of AO-labeled macrophages infected with either R or S confirms higher yields of dead phagocytes in cftr morphants at 2 dpi compared with the control embryos at 2 dpi (Figure 3E). This agrees with the presence of apoptotic immune cells seen in CF granulomatous lesions (Figure 2). Importantly, the proportion of dead macrophages was lower in WT embryos infected with S- compared with R-infected embryos (Bernut et al., 2014; Roux et al., 2016) but remained equal in the absence of CFTR (Figure 3E), substantiating the crucial role of CFTR in containing intracellular Mabs.

Modulation of the Neutrophilic Response to M. abscessus by CFTR

The chronic infections with a neutrophilic inflammation are a hallmark of CF lung pathophysiology (Cantin, 1995). Having reported that Mabs lesions are characterized by an influx of neutrophils and that these cells are critical in the host defense against Mabs infections (Bernut et al., 2014, 2016a; Malcolm et al., 2018), we next addressed whether CFTR ablation influences the behavior of neutrophils by examining and comparing the dynamic of leucocyte mobilization in WT and CFTR-deficient larvae using the Tg(mpx:GFP)i114 transgenic line labeling neutrophils with GFP (Renshaw et al., 2006). Surprisingly, CFTR deficiency strongly reduced neutrophil mobilization toward the infection sites, upon local injection of either R or S variants, as revealed by microscopy observations and quantitative analysis of the number of neutrophils at the site of infection at 4 h post-infection (hpi) in cftr morphant (Figures 4A–4D) and despite a larger baseline number of neutrophils in the absence of CFTR (Figures S3A and S3B). These phenotypes are in line with the impaired neutrophil trafficking in cftr morphants infected with P. aeruginosa (Phennicie et al., 2010).
Additionally, confocal microscopy unraveled impaired neutrophil mobilization around and into Mabs-granulomatous lesions (Figures 4E and 4F) and abscesses (Figure 4G) in the cftr morphants. We previously established the linear relationship between the number of recruited neutrophils and the Mabs granuloma volume in WT ZF (Bernut et al., 2016a). Although WT granulomas contained numerous neutrophils distributed into and on the periphery of these cellular structures, CF granulomas were dominated mostly by macrophages and contained fewer neutrophils (Figures 4E and 4F).

CF neutrophils remain capable of engulfing bacteria similarly to WT neutrophils (Figure S4A), suggesting that their phagocytosis activity toward Mabs is independent of CFTR. However, they fail to control intracellular Mabs (Figure S4B), leading to increased cell death (Figure S4C), as reported for macrophages (Figure 3). To determine whether the impaired neutrophilic response to Mabs infection in cftr-deficient ZF is linked to a possible intrinsic alteration in neutrophil recruitment, we performed a neutrophil mobilization assay using fMLP, a synthetic neutrophil chemoattractant. Injection of fMLP into the otic vesicle induced similar neutrophil recruitment in cftr morphants and control larvae (Figure S5A).

Previous findings highlighted also the requirement of IL-8 for neutrophil trafficking during Mabs infections and its crucial role in elaborating Mabs granuloma (Bernut et al., 2016a). That CFTR impairs the early mobilization of neutrophils into Mabs lesion led us to enquire whether CFTR depletion influences cxcl8 expression. qRT-PCR analysis revealed a similar level of cxcl8 expression in cftr morphants and control animals infected with both S and R variants (Figure S5B), indicating that impaired neutrophil trafficking is not caused by an alteration in the Cxcl8 pathway-mediated neutrophil mobilization.

Overall, these results suggest CFTR is required for early and late neutrophil recruitment to localized Mabs infection and to control the intracellular growth of Mabs.

**Intracellular Killing of M. abscessus Is Mediated by NOX2-Dependent ROS Production**

Mabs induces an oxidative stress response with the generation of intracellular reactive oxygen species (ROS) by macrophages and neutrophils (Bernut et al., 2016a; Malcolm et al., 2018). Loss of functional CFTR reduces the macrophage respiratory burst response and impairs killing of intracellular B. cenocepacia (Assani et al., 2017). Thus, intracellular ROS generation in CFTR-deleted larvae was investigated as a plausible mechanism through which CFTR-mediated oxidative stress controls Mabs infections. Because production of intracellular ROS by professional
phagocytes relies mainly on nox2 (Warnatsch et al., 2017), knockdown experiments abolishing initial NOX2-mediated ROS production (Figure 5A) were conducted using a specific MO targeting nox2 (Roca and Ramakrishnan, 2013). Although injection of nox2-MO failed to affect early macrophage mobilization into the HBV (Figure 5B) or macrophage phagocytosis (Figure 5C), it did lead to larger numbers of heavily infected cells (Figure 5D). Consistent with these findings, global mycobacterial loads increased in the absence of NOX2 (Figure 5E). Consistently, the nox2 morphants developed more abscesses than the control embryos, leading to the premature larval death (data not shown). This implies that reduced ROS production in nox2 morphants is deleterious for the host and that the enhanced susceptibility of these fish to the infection highlights the key role of the NADPH-mediated intracellular ROS for clearing Mabs.

To determine whether an impaired oxidative defense is involved in defective CFTR-associated reduced bacterial killing in early infection stages, we examined whether heat-killed and intact Mabs induce intracellular ROS production in macrophages using the CellROX dye. ROS-labeled phagocytes were rapidly detected, but the number of ROS-positive macrophages harboring either living or heat-killed R or S variants was lower in cftr morphant than in control ZF at 2 hpi (Figures 6A and 6B), consistent with the altered ROS production reported in the CF context (Assani et al., 2017; Phennicie et al., 2010). Of note, the larger number of ROS-positive macrophages infected with heat-killed bacteria suggests that Mabs has developed mechanisms to overcome the host oxidative killing mechanisms. Moreover, similar results were observed regarding reduced ROS production in CF-infected neutrophils (Figures S6A and S6B). To further inquire whether the impaired intracellular ROS production in absence of CFTR is linked to a possible decrease in NADPH oxidase activity, the NOX2-mediated ROS signaling pathway was dissected in defective cftr embryos. Mabs infection triggers an upregulation of nox2 in control fish and qRT-PCR analysis confirmed a reduced nox2 expression in cftr morphants (Figure 6C), suggesting that CFTR orchestrates the early regulation of ROS induction by modulating the NOX2/NADPH oxidase activity.

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That the intracellular bacterial profiles were similar in the cftr morphants and in the double cftr/nox2 morphants (Figure 6D) suggests that the enhanced intracellular bacterial growth in cftr morphants is directly linked to defective NOX2-NADPH oxidase activity.
oxidase-related ROS production. Overall, these results demonstrate that CFTR deficiency alters NOX2/NADPH oxidase-dependent ROS production that, in turn, fails to restrict intracellular growth of Mabs and that the release of NOX2-derived ROS participates in clearing Mabs in both macrophages and neutrophils (Figures 6 and S6).

Activated macrophages restrict mycobacterial growth through a TNF-mediated ROS-dependent pathway (Bernut et al., 2016a; Dewas et al., 2003). Combined with the fact that CFTR dysfunction is associated with alterations in the innate immune regulation network and pro-inflammatory cascades (Döring and Gulbins, 2009; Cantin, 1995), we next explored whether abnormal tnf induction could be involved in reduced ROS-mediated bacterial killing in cfr morphants during the early stages of infection. To assess the effect of cfr loss of function on tnfα production by Mabs-infected macrophages, Mabs E2-Crimson were injected into the muscle of either controls or cfr morphants tnfα:GFP-F/ mpeg1:mCherry-F double transgenic. Microscopy observations indicate that both WT or CF animals exhibited equal proportions of GFP-positive infected phagocytes containing either Mabs S or R at 4 hpi (Figures S7A and S7B), suggesting that the early impaired ROS generation associated with CFTR ablation is TNF independent. At later stages, however, CFTR ablation triggers a hyper-inflammatory response following infection with Mabs, with qRT-PCR revealing upregulation of tnfα expression, especially after infection with the R form (Figure S7C), similar to findings reported previously in mice (Catherinot et al., 2009).

Collectively, these data indicate that NOX2/NADPH oxidase-dependent ROS production by infected phagocytes represents a critical host defense mechanism against Mabs and suggest that the inherent deficit in NOX2-derived oxidative stress in CF leukocytes is responsible for their defective bacterial killing responses.

**DISCUSSION**

Pulmonary disease is the leading cause of morbidity and mortality in CF and is characterized by a vicious circle of chronic infections and persistent inflammation. Among the deleterious bacteria found in CF airway, the fast growing multidrug-resistant Mabs has emerged as an important respiratory pathogen of major concern in CF centers worldwide (Parkins and Floto, 2015). However, our understanding of the particular vulnerability of CF patients to Mabs infection remains limited by the lack of suitable animal models mimicking the immune abnormalities found in the CF population. Nevertheless, important insights into the pathophysiology of Mabs diseases have recently been obtained in the ZF (Bernut et al., 2014, 2016a), and the very close structural relatedness between ZF and human CFTR emphasizes further the relevance of ZF to study CFTR functions (Zhang and Chen, 2016; Liu et al., 2017). To address these unmet needs, we exploited here CF ZF as an innovative vertebrate recapitulating aspects of CF immuno-pathogenesis. Thanks to genetic and high-resolution imaging approaches, we report the direct stepwise dissection of Mabs infection in an animal lacking CFTR to elucidate the biological implication of CFTR in innate immunity to Mabs.

Mabs-infected CFTR-depleted ZF rapidly succumb to infection, reflecting a hypersusceptibility to this mycobacterium in CF, providing a first glimpse into CFTR-mediated host defenses...
to Mabs infection. Mechanisms leading to the formation of protective Mabs granuloma depend on efficient macrophage and neutrophil cooperation, orchestrated by fine-tuning innate immune responses (Bernut et al., 2016a). The spatiotemporal events associated with CFTR ablation (Figure 7) reveal a mechanism whereby CFTR participates in neutrophil chemotaxis to the infected sites and the adjustment of oxidative host defenses, conditioning efficient phagocyte-mediated bacterial killing, together generating a protective granulomatous response. Infection of ZF with Mabs is characterized by (1) rapid engulfment of the bacilli by macrophages; (2) activation of macrophages, resulting in chemotaxis guiding neutrophils to pre-forming granulomas and ROS production by NOX2 for intracellular killing of Mabs; and (3) homeostatic granuloma formation to sequester Mabs, containing the infection and favoring the development of chronic disease. Conversely, CFTR deletion promotes increased susceptibility to Mabs infections, correlating with (1) deficiency in ROS production altering phagocyte-mediated Mabs killing, resulting in increased intracellular bacterial loads and premature cell death, and (2) impaired neutrophil chemotaxis toward nascent granulomas. These two factors conspire to alter the maintenance of protective granuloma with uncontrolled extracellular mycobacterial spread, conducting to acute infection and larval death. Other studies suggested that dysfunction of CFTR dampens the microbicidal activity of immune cells (Assani et al., 2017; Di et al., 2006; Duranton et al., 2012), promoting infectious pathology in CF airways. However, existing CF models have failed to reproduce the hypersusceptibility phenotype associated with mycobacterial infections in CF (Roux et al., 2016; Le Moigne et al., 2015). Using the CF ZF model, we report here that primary alterations in innate immunity directly contribute to increased susceptibility to the infection. Whereas ROS produced by the NADPH oxidase during the respiratory burst participate in the elimination of pathogens, Mabs has been reported to withstand the hostile oxidative environments inside phagocytes (Oberley-Deegan et al., 2010), although this awaits in vivo confirmation. In ZF, inhibition of the NOX2/NADPH oxidase pathway enhances intracellular growth. Given the importance of NOX2-derived ROS production in Mabs killing, a reduced oxidative response in CF ZF is very likely to explain the increase susceptibility to Mabs. Of note, the bacterial burden in nox2-defective macrophages is lower than in CFTR macrophages (Figure 6D), suggesting that additional CFTR-mediated mechanisms are likely to participate in Mabs clearance.

Overly exuberant neutrophil influx associated with harmful oxidative stress is a hallmark of the inflammatory CF lungs (Cantin, 1995; Hector et al., 2014). The increased number of neutrophils in cftr morphants mirrors the neutrophilia seen in CF. Our results emphasize also the neutrophil chemotaxis impairment to Mabs, as shown previously in P. aeruginosa-infected ZF (Phennicie et al., 2010) but raising also controversial questions regarding inflammatory and infectious CF pathologies. Supporting the view that neutrophilic inflammation-mediated Mabs infection plays a critical role in host defense against this pathogen by maintaining granuloma integrity and preventing extracellular bacterial multiplication (Bernut et al., 2016a), we show that the capacity of neutrophils to migrate in a CFTR-dependent manner is involved in the formation of protective granulomas. Release of ROS by epithelial cells through NOX2/NADPH oxidase has been implicated in neutrophil chemotaxis to wounds (Braunersreuther et al., 2013). We provide here evidence for CFTR in modulating the NOX2 oxidative pathway, in which a
local redox imbalance in cftr morphants may account for the reduced number of infiltrated neutrophils toward Mabs. This suggests that NOX2-dependent ROS production from activated leucocytes triggers an oxidative environment sustaining neutrophilic mobilization to efficiently contain bacteria within homeostatic granulomas. The NOX/DUOX family NADPH oxidase-mediated oxidative defenses play a critical role to control invading pathogens by triggering neutrophil chemotaxis to infected tissues or bacterial killing (Brothers et al., 2013; Rada and Leto, 2008). In addition to the reduced nox2 expression in absence of CFTR, presumably contributing to imbalanced adjustments of redox signaling, other mechanisms altering ROS production via other NADPH oxidase complexes by epithelial or immune cells may also contribute to the impaired bactericidal function and/or chemotaxis of leucocytes during Mabs infection.

The clinical relevance of ROS production in host defense, notably the NOX2/NADPH oxidase in granuloma formation, is consistent with mutations in nox2 leading to chronic granulomatous disease (CGD) typified by the development of large size and poorly structured granulomas that are unable to sequester mycobacteria (Deffert et al., 2014) and associated with severe inflammation (Rieber et al., 2012). However, although the altered NADPH oxidase function in CGD can lead to increased disease severity following infection with M. tuberculosis or vaccination with M. bovis BCG (Deffert et al., 2014), reports of infection with NTM remain anecdotal (Ohga et al., 1997; Weening et al., 2000; Chusid et al., 1975), and no particular link with M. abscessus has been ascribed yet.

This, together with clinical cases of CF patients heavily infected with Mabs R (Jönsson et al., 2007; Catherinot et al., 2009), demonstrates that Mabs exacerbates inflammation in absence of CFTR, implicating a critical inflammatory pathology associated with tissue damage and persistent Mabs infections. Thus, at the later stages of infection, it is possible that an imbalance of ROS production or neutrophil chemotaxis could be caused by CF-mediated hyperinflammation acting as a negative feedback loop that would undo the fine-tuning of immune responses.

Our study indicates that cftr is a regulator of host immunity to MABSC but not to other saprophytic (M. smegmatis) or pathogenic (M. marinum) NTM, suggesting that species-specific restriction mechanisms may exist for these organisms. In fact, we show that M. marinum-infected cftr morphants succumbed to infection more slowly than controls, suggesting that inactivation of cftr triggers a mild protective immunity against tuberculosis. Indeed, it has been proposed that the high carrier rate for CFTR in M. tuberculosis infection (Bosch et al., 2017). This species-specific susceptibility to different mycobacteria is particularly intriguing and deserves further attention.

In summary, we demonstrate that CFTR dysfunction leads to hypersusceptibility to Mabs infection in vivo, potentially explaining the high rates of infection seen clinically in CF patients. We anticipate that insights obtained from ZF may guide the development of future therapies targeting innate immune defects in CF.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes one table and seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2019.01.071.

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**AUTHOR CONTRIBUTIONS**

A.B. and L.K. conceived the study and wrote the manuscript with input from J.-L.H., R.A.F., and S.A.R., A.B., S.A.R., and L.K. designed experiments and analyzed data. A.B. performed experiments and produced the figures. C.D. performed PCR experiments. N.V.O. made and provided the Tg(mpeg1:NLSclover)sh436 line. A.N. performed EM preparation and imaging experiments. All authors reviewed the manuscript.

**DECLARATION OF INTEREST**

The authors declare no competing interests.
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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental Model: Bacterial Strains** | | |
| *Mycobacterium abscessus sensu stricto*, strain CIP104536<sup>T</sup>, smooth | Laboratoire de Référence des Mycobactéries (IP, France) | ATCC19977<sup>T</sup> |
| *Mycobacterium abscessus sensu stricto*, strain CIP104536<sup>T</sup>, rough | Laboratoire de Référence des Mycobactéries (IP, France) | ATCC19977<sup>T</sup> |
| *Mycobacterium abscessus subsp. bolletii*, CIP108541<sup>T</sup>, smooth | Laboratoire de Référence des Mycobactéries (IP, France) | CIP108541<sup>T</sup> |
| *Mycobacterium abscessus subsp. bolletii*, CIP108541<sup>T</sup>, rough | Bernut et al., 2016b | CIP108541<sup>T</sup> |
| *Mycobacterium abscessus subsp. massiliense*, CIP108297<sup>T</sup>, smooth | Laboratoire de Référence des Mycobactéries (IP, France) | CIP108297<sup>T</sup> |
| *Mycobacterium abscessus subsp. massiliense*, CIP108297<sup>T</sup>, rough | Laboratoire de Référence des Mycobactéries (IP, France) | CIP108297<sup>T</sup> |
| *Mycobacterium marinum* strain M | Stinear et al., 2008 | ATCC BAA-535 |
| *Mycobacterium chelonae* strain A6 | Laboratoire de Référence des Mycobactéries (IP, France) | N/A |
| *Mycobacterium smegmatis mc<sup>2</sup>155* | Snapper et al., 1990 | N/A |
| **Experimental Model: Zebrafish lines** | | |
| golden mutant | Lamason et al., 2005 | N/A |
| cfr<sup>potato</sup> mutant | Navis et al., 2013 | N/A |
| gBAC(cfr-RFP)pd1042 | Navis et al., 2013 | N/A |
| Tg(mp5c:GFP)<sup>114</sup> | Renshaw et al., 2006 | N/A |
| Tg(LysC<sub>DSRed</sub>)nz5 | Hall et al., 2007 | N/A |
| Tg(impe1:NLSclover)sh436 | This study | N/A |
| Tg(impe1:mCherry-F)ump2 | Bernut et al., 2014 | N/A |
| Tg(infa:GFP-F)ump5 | Nguyen-Chi et al., 2015 | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Hygromycin B | Sigma-Aldrich | Cat# H3274 |
| Tricaine | Sigma-Aldrich | Cat# E10521 |
| Difco Middlebrook 7H9 Broth | Thermo Fisher Scientific | Cat# DF0713-17-9 |
| Middlebrook OADC Growth Supplement | Sigma-Aldrich | Cat# M0678 |
| Tween-80 | Sigma-Aldrich | Cat# P1754 |
| f-Met-Leu-Phe (fMLP) | Sigma-Aldrich | Cat# F3506 |
| Acridine Orange | Invitrogen | Cat# 93001 |
| CellROX Deep Red | Invitrogen | Cat# C10422 |
| LR Clonase II Plus | Invitrogen | Cat# 12538 |
| SuperScript IV First-Strand Synthesis System | Invitrogen | Cat# 18091050 |
| LightCycler® 480 SYBR Green I Master | Roche | Cat# 04887352001 |
| **Recombinant DNA** | | |
| pTEC15 | Addgene | Cat# 30174 |
| pTEC19 | Addgene | Cat# 30178 |
| pTEC27 | Addgene | Cat# 30182 |
| pcDNA3.1-Clover-mRuby2 | Addgene | Cat# 49089 |
| Gateway pDONR221 Vector | Invitrogen | Cat# 12536017 |
| pcSTP | Kawakami et al., 2004 | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Laurent Kremer (laurent.kremer@irim.cnrs.fr).

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Bacterial Strains

Mycobacterial strains carrying pTEC15 (Addgene, plasmid 30174), pTEC27 (Addgene, plasmid 30182) or pTEC19 (Addgene, plasmid 30178) that express green fluorescent protein (Wasabi), red fluorescent protein (tdTomato) or bright far-red fluorescent protein (E2-Crimson), respectively, were grown under hygromycin B selection in Middlebrook 7H9 supplemented with oleic acid, albumin, dextrose, catalase (OADC), and 0.05% Tween-80. To prepare heat-killed Mabs, bacteria were incubated at 80°C for 20 min.

Zebrafish Husbandry and Ethic statements

Experimental procedures were performed using the golden mutant (Lamason et al., 2005), the cfr (Navis et al., 2013), the transgenic lines Tg(mpx:GFP)i114 (Renshaw et al., 2006) and Tg(LysC_DSred)n25 (Hall et al., 2007) to visualize neutrophils; Tg(mpeg1:NLSclover)sh436 and Tg(mpeg1:mCherry-F)ump2 (Bernut et al., 2014) to visualize Mφ; gBAC(cfr-RFP)pd1042 (Navis et al., 2013) to visualize cfr expression, and Tg(tnfα:GFP-F)ump5 (Nguyen-Chi et al., 2015) to visualize tnf-α expression. ZF were raised and maintained according to standard protocols (Nusslein-Volhard and Dahm, 2002). Eggs were obtained from pairs of adult fish by natural spawning and raised at 28.5°C in tank water. The ZF husbandry and all ZF experiments described in the present study were conducted in accordance with guidelines from the UK Home Office (Bateson Centre, University of Sheffield) and in compliance with the European Union guidelines for handling of laboratory animals (CNRS, Montpellier) and were approved by the Direction Sanitaire et Vétérinaire de l’Hérault et Comité d’Ethique pour l’Expérimentation Animale de la région Languedoc Roussillon under the reference CEEA-LR-1145 (Montpellier experiments).

METHOD DETAILS

Creation of the Tg(mpeg1:NLSmClover) transgenic line

The Tol2kit multisite Gateway-based transposon system (Kwan et al., 2007) was used to generated a construct from which a stable transgenic line was raised. mClover was amplified from pcDNA3.1-Clover-mRuby2, a gift from Kurt Beam (Addgene, plasmid 49089), using forward forward primer 5’-GGGGACAAGTTTGTACAAAAAAAGGCTCAATGGCTCCAAAGAAGAAGCGTAAGGTA-3’ and reverse primer 5’-GGGGACCACTTTGTCAAAAAAAGGCTCAATGGCTCCAAAGAAGACGTAAGGTA-3’ and cloned into pDONR 221 donor vector (Invitrogen) to produce pME-NLSmClover. An LR Clonase II Plus (Invitrogen) Gateway reaction was performed with pSE-mpeg1 (Ellett et al., 2011), pME-NLSmClover and p3E-polyA inserted into pDestTol2pA2 destination vector (Kwan et al., 2007) to
produce mpeg1:NLSmClover construct. The tol2-transposase mRNA was synthesized from pCSTP (Kawakami et al., 2004) and co-injected with the mpeg1:NLSmClover construct into one-cell-stage ZF embryos to create the Tg(mpeg1:NLSmClover)sh436 transgenic line harboring green fluorescent macrophages.

**Morpholino injection**

MO were purchased from Gene Tools. cftr splice-blocking morpholino targeting cftr (ZFIN, ZDB-GENE-050517-20) (5’-GACA CATTITGGGACTGCACTCACTCAAA-3’) was injected into one-cell-stage zebrafish embryos (1 mM, 2 nl). The efficiency of gene knockdown was confirmed by RT-PCR and sequencing with the following primers for both sides of the MO target sequence (forward and reverse): CCTGTGGAGGATGCCAACTGCC and TGCATGCCCCAGTGCTGCAG. Total RNA from 2 dpf embryos (pools of 10 each) was isolated and purified with Nucleospin RNAII kit (Macherey-Nagel), following the manufacturer’s instructions. The SuperScript IV reverse transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo(dT) primer from 1 g of total RNA at 50 °C for 50 min. MO for nox2 (cybb, ZFIN, ZDB-GENE-040426-1380) knockdown (5’-CATAATCCGATAGCTTACGATAAC-3’) was prepared and injected as described earlier (Roca and Ramakrishnan, 2013). A standard control-MO was included as a negative control in all experiments.

**Zebrafish infection**

Bacterial inoculate were prepared for infection challenges in ZF embryos/larvae, according to procedures described earlier (Bernut et al., 2015). Briefly, systemic infections were carried out by the injection of single-cell suspensions of known titer (100-200 colony-forming units) into the caudal vein of 30 hpf embryos. For leucocyte mobilization assays, mycobacteria were locally injected into the hindbrain ventricle at 30 hpf, the otic vesicle or the muscle compartment of 3 dpf larvae. The number of animals used for each procedure was guided by pilot experiments or by past results (Bernut et al., 2016a; Bernut et al., 2014).

**qRT-PCR**

Total RNA was prepared and first-strand cDNA synthesized. Real-time RT-PCRs were performed with an LightCycler® 480 system instrument using LightCycler® 480 SYBR Green I Master (Roche) and gene expressions were detected with gene-specific primers listed in Table S1. Reaction mixtures were incubated for 5 min at 95 °C, followed by 45 cycles of 5 s at 95 °C, 20 s at 65 °C, and finally 10 s at 95 °C. Each experiment was run in triplicate. qRT-PCR data are analyzed with the LightCycler® 480 Software, normalized to the housekeeping gene ef1α and calculated using the ΔΔCt method.

**Epifluorescence, Confocal Microscopy and Imaging**

To quantify bacterial loads, granulomas (defined as cellular aggregates comprising at least 10 infected cells), cords, leucocyte mobilization, and evaluate oxidative stress, infected larvae were tricaine-anesthetized and mounted in 0.8% low melting point agarose with 0.016% tricaine for real-time microscopy observations. To evaluate intracellular mycobacterial growth, phagocytosis, mobilization, and evaluate oxidative stress, infected larvae were tricaine-anesthetized and mounted in 0.8% low melting point agarose with 0.016% tricaine for real-time microscopy observations. To evaluate intracellular mycobacterial growth, phagocytosis, mobilization, and evaluate oxidative stress, infected larvae were tricaine-anesthetized and mounted in 0.8% low melting point agarose with 0.016% tricaine for real-time microscopy observations. Epiﬂuorescence microscopy was performed using a Zeiss Plan Neo Fluor Z 1x/0.25 FWD objective and equipped with an AxioCam503 monochrome (Zeiss) camera. Pictures were taken and processed using ZEN 2 (blue edition). Confocal microscopy was performed using a Spinning disc confocal Perkin Elmer Ultraview VoX inverted Olympus IX81 with a UplanSAPO 20x/0.8 and a UplanSAPO 40x/1.3 oil objective, equipped with a Hamamatsu C9100-50 EM-CCD camera or with a Leica SPE upright microscope with a ACS APO 40x/1.5 oil objective. Images were captured and processed using the Velocity or LASAFS softwares.

**Transmission Electron Microscopy**

For TEM, ZF larvae were tricaine-anesthetized and fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1M phosphate buffer. After a post-fixation in 1% osmic acid 1 hr at 4 °C and 0.5% tannic acid for 30 min at 4 °C, animals were dehydrated in successive ethanol baths, infiltrated with mixes of epon 812/propylene oxide, embedded in epon 812 resin, and then polymerized at 60 °C for 48 hr. Ultrathin sections were cut with a Reichert Ultracut ultramicrotome (Leica) and collected on nickel grids. Finally, the grids were stained 20 min in 2% uranyl acetate and 3 min in 3% lead citrate, then examined on a Tecnai G2 F20 (200kV, FEG) electron microscope. Images were captured using TIA imaging software.

**Neutrophils Recruitment Assay**

Neutrophil mobilization was elicited through injection of 300 nM f-Met-Leu-Phe (fMLP) chemoattractant into the otic cavity of 3 dpf larvae (Bernut et al., 2016a) and counting the recruited neutrophils at the injection site using fluorescence microscopy. 

**Cell Death and Reactive Oxygen Species Detection**

Leucocyte killing in ZF was detected using Acridine Orange (AO), as previously described (Bernut et al., 2014). Living embryos were soaked in 10 mg/ml AO in fish water for 30 min at 28.5 °C, followed by two washes, then replaced at 28.5 °C until observation and...
analyzed by confocal microscopy. For ROS detection, living larvae were soaked in 5 μM CellROX Deep Red Reagent in PBS for 30 min at 28.5°C, rinsed twice with PBS and directly prepared for confocal microscopy observations.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis (Prism 7.0; GraphPad Software or R 3.5.0) was performed using χ²/Fisher’s exact test, two-tailed unpaired Student’s t test for comparisons between two groups and one-way or two-way ANOVA (with appropriate post-test adjustment) for other data. Mantel-Cox Log-rank test was used to compare survival curves. All data are plotted as average of two or three independent experiments. All error bars indicate standard errors of means (SEM). ns, not significant (p ≥ 0.05); *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.