CHAPTER TWO

Control of mitochondrial functions by *Pseudomonas aeruginosa* in cystic fibrosis

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Abstract

Cystic fibrosis (CF) is a genetic disease characterized by mutations of cystic fibrosis transmembrane conductance regulator (CFTR) gene, which lead to a dysfunctional chloride and bicarbonate channel. Abnormal mucus viscosity, persistent infections and hyperinflammation that preferentially affect the airways, referred to the pathogenesis of CF lung disease. It has largely demonstrated that *Pseudomonas aeruginosa* (*P. aeruginosa*) represents the most important pathogen that affect CF patients, leading to worsen inflammation by stimulating pro-inflammatory mediators release and tissue destruction. The conversion to mucoid phenotype and formation of biofilms, together with the increased frequency of mutations, are only few changes that characterize the *P. aeruginosa*'s evolution during CF lung chronic infection. Recently, mitochondria received increasing attention due to their involvement in inflammatory-related diseases, including in CF. Alteration of mitochondrial homeostasis is sufficient to stimulate immune response. Exogenous or endogenous stimuli that perturb mitochondrial activity are used by cells, which, through the mitochondrial stress, potentiate immunity programs. Studies show the relationship between mitochondria and CF, supporting the idea that mitochondrial dysfunction endorses the exacerbation of inflammatory responses in CF lung. In particular, evidences

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suggest that mitochondria in CF airway cells are more susceptible to *P. aeruginosa* infection, with consequent detrimental effects that lead to amplify the inflammatory signals. This review discusses the evolution of *P. aeruginosa* in relationship with the pathogenesis of CF, a fundamental step to establish chronic infection in CF lung disease. Specifically, we focus on the role of *P. aeruginosa* in the exacerbation of inflammatory response, by triggering mitochondria in CF.

1. Introduction

The mitochondrion, usually termed as the "powerhouse" of the cell, is a membrane-bound organelle, which, despite other intracellular organelles, presents an inner (IMM) and an outer membrane (OMM) (Scarpulla, 2008). The pore-forming membrane proteins that characterize the OMM, allow the transfer of ions and uncharged molecules, while larger molecules require specialized protein complexes (Yang et al., 2021). At the same time, small molecules including oxygen, carbon dioxide and water may diffuse through the IMM, while macromolecules are transported by specialized translocase. Together, IMM and OMM determine two compartments, namely: intermembrane space (IMS) and matrix (Kuhlbrandt, 2015). The IMM composes numerous folds that protrude into the matrix (known as cristae) that improve the surface area, enhancing the efficiency of mitochondrial ATP production (Oyewole and Birch-Machin, 2015). Located in the IMM, the "Mitochondrial Calcium Uniporter" (MCU) complex is responsible for the mitochondrial calcium (Ca^{2+}) uptake, that plays an important role in the regulation of cellular functions, such as autophagy and apoptosis (Giorgi et al., 2018). Indeed, different carriers and dehydrogenases are activated by elevated mitochondrial Ca^{2+} level, leading to an increase of respiratory rate and ATP production required for the energy state of the cells (Patergnani et al., 2011). In the matrix, numerous complexes catalyze the reactions that convert pyruvate and fatty acid in acetyl-CoA. Subsequently, acetyl-CoA enters in the tricarboxylic acid cycle (TCA) that forms NADH and $FADH_2$, which, in turn, act as electron-carriers for the respiratory chain complexes. Situated on the IMM, the four complexes (I–IV) of mitochondrial electron transport chain (mETC) are the "generators" of electrochemical gradient required to generate ATP and for Ca^{2+} uptake into the matrix. One of the main consequences of mitochondrial respiration is the generation of unpaired electrons. During the reduction in H_2O, may happen that spurious electrons mainly originating from the complex-I (NADH-CoQ reductase) and complex III (cytochrome *c* reductase), reduces oxygen (O_2) to produce Radicals Oxygen Species

(ROS), including superoxide anion ($O_2^{\bullet -}$) (Mailloux and Harper, 2011). To protect the cell, mitochondrion contain antioxidant defenses including superoxide dismutase, peroxidases and catalase that counteract the detrimental effect of ROS (Mailloux, 2018; Oyewole and Birch-Machin, 2015).

Mitochondrial DNA (mtDNA) is a closed circle-double DNA contained into the matrix, that encodes for 37 genes including 13 components of mETC (Gammage and Frezza, 2019). Together with ROS, cardiolipin and ATP, mtDNA represent one of principal mitochondrial damage-associated molecular patterns (mtDAMPs). Mitochondrial "danger" signals that activate pattern recognition receptors and trigger intracellular signal cascades, which, subsequently, prompt a pro-inflammatory mediators release through the activation of inflammasomes (Grazioli and Pugin, 2018; Patergnani et al., 2021).

Maintaining the mitochondrial homeostasis is fundamental for the cellular health. Several evidences have demonstrated that the mitochondrial dysfunction contributes significantly to worsen the pathogenesis of inflammatory-related diseases, including cystic fibrosis (CF). About this, different mitochondrial quality control processes, such as mitochondrial fusion complementation, mitophagy, mitochondrial unfolded protein responses (UPR^{mt}) and apoptosis, are involved to preserve and/or recovery the mitochondrial homeostasis under stress conditions, such as the recurrent pathogen infections. Mitochondrial fusion compensation allows the exchange of materials among partially damaged mitochondria, improving the functions of organelle (Sabouny and Shutt, 2020). Furthermore, prolonged stress conditions promote the isolation of mitochondrial damaged member in autophagosomes that, through lysosomal enzymes, will be eliminated by mitophagy. This pathway leads to remove excess of ROS and others dangerous factors in order to minimize the dysfunctional mitochondria (Eisner et al., 2018). The recovery of mitochondrial function is also guarantee by UPR^{mt} activation, a mitochondrial-nuclear mechanism that responds to stress stimuli by improving the transcription of nuclear genes, encoding mitochondrial chaperones and proteases (Zhou et al., 2022). As well known, *Pseudomonas aeruginosa* (*P. aeruginosa*) induces UPR^{mt} through the recruitment of stress-activated transcription factor 1 (ATFS-1), which stimulates the transcription of mitochondrial chaperones [heat shock protein (HSP) 10 and HSP 60], proteases and immune genes in *C. elegans* (Pellegrino et al., 2014). While in resting conditions ATFS-1 is imported into mitochondria to be degraded by the Lon protease 1, during mitochondrial dysfunction the mitochondrial protein import system is impaired, favoring the

nuclear ATFS-1 translocation. Apoptosis is another important mitochondrial stress response activated during irreversible damages to declare the cell fate (Green and Kroemer, 2004). The prolonged stress environment provoked by harmful stimuli or pathogens induces mitochondrial potential ($\Delta\psi$) depolarization, membrane permeabilization, release of pro-apoptotic factors and activation of caspases (Bonora et al., 2022; Green and Kroemer, 2004; Wood et al., 2015).

In this review, we highlight the role of the main bacterium involved in CF lung disease, *P. aeruginosa*, focusing on its evolution and adaptation in CF pathogenesis. We will discuss the impact of *P. aeruginosa* on mitochondria, underlying the detrimental effects on mitochondrial homeostasis, which, in turn, exacerbates the inflammatory responses in CF lung.

2. The physiopathology of cystic fibrosis

CF is an autosomal recessive disease caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, localized on chromosome 7 (Kerem et al., 1989; Rommens et al., 1989). CFTR channel is an ATP-dependent transporter of chloride and bicarbonate, it is regulated by protein kinase A and C phosphorylation. Typically expressed on the apical membrane of several epithelial cells, the airway secretory cells are the dominant CFTR-expressing cell type (Carraro et al., 2021; Okuda et al., 2021); CFTR extrudes anions to control the hydration level of biological secretions. In addition to direct ion transport, CFTR channel inhibits the activation of the epithelial sodium channel (ENaC) (Gentzsch et al., 2010) and regulates other chloride channels (Gabriel et al., 1993).

Despite some mutations display various defects, such as F508del-CFTR, with defective trafficking, function, and stability (Veit et al., 2016). CFTR mutations have been categorized into six major classes depending on their main pathologic effects on CFTR protein: class I (absence of protein expression), class II (protein trafficking defect), class III (no function), class IV (reduced function), class V (reduced protein expression) and class VI (reduced protein stability) (Bell et al., 2015). This classification may be useful to advise therapy for patients, as different therapies have been currently evolved that target CF disorder by distinct classes of CFTR mutations (Chen et al., 2021).

CF is considered a multiorgan disease, several organs are affected, such as exocrine pancreas, liver, intestinal tract, salivary glands and reproductive organs, but the most affected is the respiratory tract. It represents the main

cause of morbidity and reduction of life expectancy in CF patients (Shteinberg et al., 2021).

The defective extrusion of chloride and bicarbonate and the excessive sodium reabsorption resulting by CFTR gene mutations alter the airway surface liquid (ASL) residing on top of CF airway epithelial cells. The ASL is a hydro-gel mucus layer with gel-forming mucins constituting a mesh that traps dust and microorganisms. These microorganisms are rapidly transported from distal airways to trachea by ciliary beating, resulting the muco-ciliary clearance the basic innate defense mechanism in the lungs. The pH and the relative content of mucins and water in ASL are important parameters to control the degree of viscosity of the mucin hydro-gel (Haq et al., 2016). In CF, dehydration reduces the periciliary liquid and increases the viscosity of the mucin hydro-gel mesh, which reduces the frequency of the muco-ciliary beating mechanism (Boucher, 2019). Adequate swelling and hydration of gel matrices are essential to maintain proper luminal and ductal transport of released mucin. It has been demonstrated that the absence of bicarbonate or the inhibition its transport reduced the amount and the rate of mucus release, like the inhibition of CFTR activity. Bicarbonate seems to be essential for extracellular mucin expansion and solubilization by reason of its ability to sequester Ca^{2+} and H^{+}, which displays the repulsive electrostatic forces of polyanionic mucins and/or separates different mucin interactions (Quinton, 2008). Therefore, bicarbonate may provide to avoid the production of aggregated mucus that is slow to release from the surfaces of the intestines or female reproductive tract (Garcia et al., 2009; Muchekehu and Quinton, 2010).

The beginning of CF lung disease has been demonstrated since the early months of the life of CF infants. Lung disease starts from a clinically asymptomatic phase that is followed by recurrent, and lately, chronic bacterial infections, associated with pulmonary inflammation. These conditions lead to damages of the bronchial walls, with dilatations (bronchiestasis) filled with mucopurulent sputum. A huge number of microorganisms and polymorphonuclear neutrophils (PMNs) in lumen constitutes an infective/inflammatory condition leading to progressively severe obstructive respiratory insufficiency (Stoltz et al., 2015). Several studies have reported that altered expression and function of CFTR channel also impairs the intracellular Ca^{2+} homeostasis, which it is normally regulated by systems of Ca^{2+}-entry and Ca^{2+}-efflux located in the plasma membrane and organelles, including mitochondria. The dysregulation related to these systems also contributes to exacerbate the inflammatory responses in CF lung, this will be argument in the following sections (as reviewed in (Rimessi et al., 2021)).

Recurrent pulmonary infections and hyperinflammation are the two most important aspects that characterize the pathophysiology of CF lung disease. In early childhood, patients are more prone to recurrent *Haemophilus influenzae* (*H. influenzae*) and *Staphylococcus aureus* (*S. aureus*) infections. In adulthood, the bacterium that most infects the CF airways is *P. aeruginosa*, that causes chronic infections, which, in turn, represent a major risk of increased severity of the disease. The cellular and humoral immune defenses of CF patients are inefficient in eliminating bacterial infections. Consequently an "exaggerated" inflammatory response is generated, characterized by an enormous amount of PMN filling the lumen of the airways. Both recurrent and chronic infections and the excessive inflammation contribute to tissue damage of the bronchial wall and progressive obstruction of airway flow, leading to progressively severe respiratory failure (Stoltz et al., 2015). The activity of the innate inflammatory immune response in the CF lungs is an issue that it has been debated for many years, and it will be argument in different sections of this review. However, it is known that bacterial infection occurs very early in the life of CF, inducing or amplifying a series of inflammatory responses of the host modulated by different cellular components of the mucosa (Roesch et al., 2018). Several hypotheses have been proposed to explain the CF pulmonary predilection for *P. aeruginosa* infection and excessive inflammation. It has been shown that following infection, ROS release contributes to a pronounced pro-oxidative redox imbalance (Galli et al., 2012). In addition, the diffusion of DNA on the mucosal surface, both by necrosis of the PMN and by the release of extracellular neutrophil traps (NETs), further increases the viscosity of the ASL and contributes to worsening the severity of lung disease (Keir et al., 2021). An excessive production of NETs has been reported in CF neutrophils, caused by their prolonged survival that is associated with the absence of CFTR function. It has been demonstrated that the "pro-survival" phenotype of CF neutrophils is assignable to delay of apoptosis, probably as primary defect in CF neutrophils (Gray et al., 2018). As a result, augmented NET formation represents another important pro-inflammatory stimulus together with protease exocytosis that contribute to damage the fibers of the extracellular matrix of the bronchial walls with very limited effects on bacterial killing (Clancy et al., 2018). CF patients' airways are characterized by abnormal infiltration of neutrophils, which are able to release abundant proinflammatory chemokines and cytokines, such as interleukin-8 (IL-8) and IL-1β, respectively (Bruscia and Bonfield, 2016a). In addition, the hyperinflammation observed in CF lung is in part due by altered phenotype of this airway immune cells associated to defective CFTR. Of note, neutrophils in

CF airways, exhibit altered chlorination of phagocytosed bacteria, that worsening the pulmonary injury. Mutations in CFTR gene are also implicated in the immune functions of macrophages, which show a reduced efficiency in the removal apoptotic cells, including dead neutrophils, a critical step for restoring the tissue homeostasis (Bruscia and Bonfield, 2016b; Vandivier et al., 2002). Overall CF macrophages preferentially adopt a proinflammatory state through the polarization to M1 phenotype due to pro-inflammatory environment that arises, characterized by high levels of IL-8, IL-1β, IL-6 and Tumor necrosis factor (TNF-α), and low levels of IL-10, but also by inability to repolarize in an M2 (anti-inflammatory) phenotype (Assani et al., 2014).

Finally, the chronic infective/inflammatory condition has implications not only on bronchial wall but also on the efficiency of rescue of mutant CFTR in CF patients treated with novel CFTR modulators. The new therapy in CF aim to correct the basic defect of CFTR administrating correctors and potentiators, which intervene to modulate the expression and function of mutated CFTR protein in plasma membrane. Several studies have already shown that their efficiency is significantly reduced during a bacterial infection mediating the recruitment of different mechanisms that include: ROS, quorum sensing, LasB protease, CFTR scaffold protein (NHERF1) and kinases (Mixed Lineage Kinase-3) (Cabrini et al., 2022; Rubino et al., 2014; Saint-Criq et al., 2018; Swiatecka-Urban et al., 2006). In contrast, the exposure to "mild insults," such as supernatant of mucopurulent material from CF patients, showed increased F508del-CFTR rescue after Trikafta administration in vitro, which appeared to have been enhanced by airway inflammation (Gentzsch et al., 2021). However, it should be emphasized that this is a mild inflammation, not caused by proliferating bacteria. Thus, the chronical susceptibility to lung infections remains an obstacle for the new CF therapy, as confirmed by results obtained administrating the antibiotic tobramycin or the antimicrobial peptide, 6K-F17, that completely restored the ORKAMBI®-mediated rescue of F508del-mutated CFTR protein expression in bronchial epithelial cells infected with *P. aeruginosa* (Laselva et al., 2020).

3. *P. aeruginosa* is the major pathogen in the CF

P. aeruginosa, a gram-negative, opportunistic, ubiquitous environmental bacterium, has become the predominant infectious agent in CF, leading cause of morbidity and mortality in CF patients. Although the

pulmonary infection in CF has been always considered polymicrobial, *P. aeruginosa* is the major responsible for chronic infection in CF lungs, in which many different strains of *P. aeruginosa* are effectors of lung tissue damage (Fig. 1) (Haq et al., 2016; Lyczak et al., 2002). While at the time of birth the CF babies's lungs are normal if compared to healthy, progressively, a chronic pseudomonal infection occurs in up to 85% of patients by adolescence, showing also an aggressive inflammatory response which

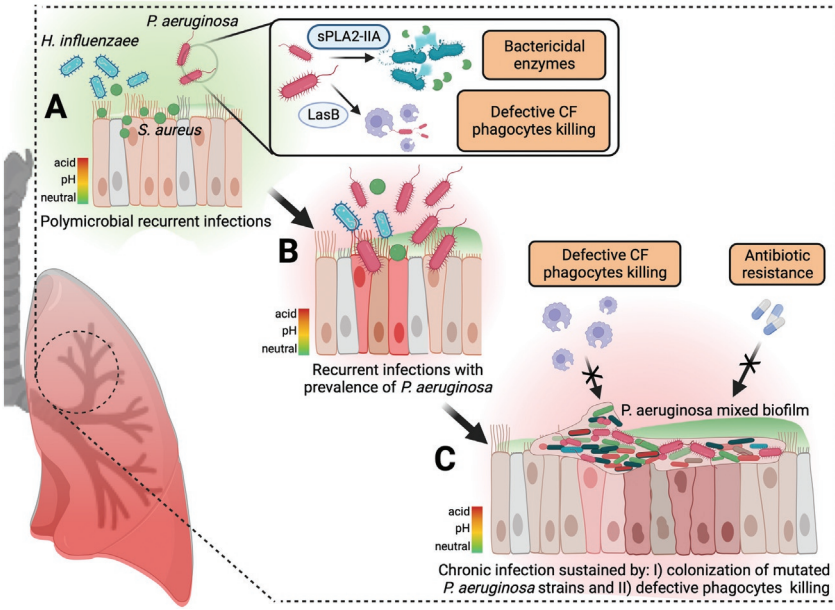

Fig. 1 Time course of *P. aeruginosa* evolution in CF lung. The defects associated to CFTR mutations lead to an alteration of the viscosity and consistency of the ASL and compromise the efficiency of the respiratory ciliary beat, impairing the mucociliary clearance in CF lung disease. Consequently, the ability of the bacterium to remain trapped inside the airways increases, and this promotes the development of inflammation from the first years of life of patients with CF. (A) In early phase of childhood a planktonic, free flowing flagellated and piliated *P. aeruginosa* substitutes *H. influenzae* and *S. aureus* in the recurrent infections in CF lung, promoting the host secretion of bactericidal enzyme type-IIA-secreted phospholipase A2 and the release of protease LasB. (B) During adolescence, the presence of polymicrobial bacterial populations is progressively reduced and frequent infections are characterized by a greater presence of *P. aeruginosa*. (C) In the adulthood of the CF patient, the wt genome of *P. aeruginosa* undergoes a series of mutations that lead to: (i) loss of pili and flagellum; (ii) envelop multidrug resistance systems; and (iii) favor the construction of biofilm to favor the growth and the protection of bacteria.

is implicated in the progression of lung damage (Armstrong et al., 1995; Chow et al., 1982; Oppenheimer and Esterly, 1975). Only a smaller number of patients will become infected with other pathogens, such as *Burkholderia cepacian*, *S. aureus* and/or non-tuberculous mycobacteria.

Several hypotheses have been suggested to justify the high rate of *P. aeruginosa* prevalence associated to the basal CFTR defects emerge until now. The mucus abnormality and the impaired mucociliary clearance constitute a favorable milieu for bacterial growth while the enhanced availability of bacterial receptors, the perturbed ingestion and reduced defense of CF phagocytes contribute to permanence of pathogens in the CF lung, resulting in critical conditions to establish the pathogen infection (Esther et al., 2019). The ASL composition facilitates the bacterial trapped favoring the contact time with the airway cells, promoting inflammation (Smith et al., 1996). The hyperabsorption of sodium and water depletes the volume of ASL, compromising the efficiency of ciliary beat respiratory (Matsui et al., 1998). Also, the high-salt level and the change of pH in ASL may participate to mucociliary impairment in CF lung disease (Ballard et al., 1999; Knowles et al., 1997; Smith et al., 1996). Several antibacterial defense proteins present in the ASL are salt-sensitive, such as β-defensis, lysozyme and lactoferrin, while phagocytic and mucociliary functions may be sensitive to acidic environment. Indeed, the defective interaction between mutant CFTR and the tumor suppressor Phosphatase and Tensin homolog deleted in chromosome 10 (PTEN) induces mitochondrial metabolic dysfunction with consequent increase in itaconate and succinate release, metabolites that promote a selective advantage for *P. aeruginosa* infection in CF (Riquelme et al., 2017, 2019).

A planktonic, free flowing flagellated and piliated *P. aeruginosa* substitutes *H. influenzae* or *S. aureus* in the recurrent infections in CF lung in early phase of childhood, at a median age of 1 year (Fig.1A) (Li et al., 2005; Tang et al., 1995). *P. aeruginosa* starts to colonize the CF airways: (i) promoting the host secretion of bactericidal enzyme type-IIA-secreted phospholipase A2, that predominately targets *S. aureus*; (ii) through the release of protease LasB, which blocks the bacterial killing activity of CF phagocytes (Bastaert et al., 2018; Pernet et al., 2014). Recently, it has been demonstrated that *P. aeruginosa* diverts the host immune system toward type 2 responses to provide a significant advantage to the pathogen growth in a LasB-dependent manner, resulting in mucin production which it was used as an energy source (Agaronyan et al., 2022). Once acquired, the *P. aeruginosa* gains a foothold in the CF airway, in fact a chronic phenotypes and genotypes emerge during the recurrent infections due to ecological

adaptation to the CF lung milieu as well as intraspecies competition between different *P. aeruginosa* strains (Fig. 1B). While most *P. aeruginosa* strains isolated from CF patients in the early phase of infection are piliated and flagellated, the pathogens isolated from CF patients with chronic infection are without structures (Davies et al., 1997; Saiman and Prince, 1993). These evidences support the role of pili and flagellae for the adherence to cell surface during the first steps of infection. Abundant on the surface of CF respiratory cells, AsialoGM1 receptor has been identified as receptor able to bind both pili and flagellae (Saiman and Prince, 1993). This enhanced expression of asialoGM1 is associated with the increased adherence of *P. aeruginosa* to CF airway cells respect to non-CF (Davies et al., 1997; Saiman and Prince, 1993). The binding between pili and flagellae with asialoGM1 receptor promotes mitochondrial dysfunction and inflammasome activation with consequent release of pro-inflammatory cytokine IL-1β and of the major neutrophil attractant cytokine IL-8 from CF airway cells (Ratner et al., 2001; Rimessi et al., 2015). The evolution between motile and non-motile *P. aeruginosa* phenotype is arisen in CF patients at the median age of 13 years, where further conversions are induced to enhance the survival, the invasion and host defenses evade capacity of pathogen (Li et al., 2005). Quorum sensing (QS) refers to a mechanism for chemical communication, originally recognized as a regulatory mechanism controlling the transcription of genes associated with virulence that allows to bacteria to detect the density of a population within a given space (Fig. 1C). It has been reported that *P. aeruginosa* from chronic infections appears to frequently lose the ability to perform QS (Passador et al., 1993; Pearson et al., 1994). Indeed, the identification of mutations that reengineer the *P. aeruginosa* QS regulatory pathway, suggests that QS is not lost during infection but rather rewired. The QS system of *P. aeruginosa* is highly complex and controlled by two acyl-homoserine lactone systems and a *Pseudomonas* quinolone signal, which is mediated through several quinolone autoinducer signaling molecules (Pesci et al., 1999). The conversion to mucoid and the formation of biofilms are two of main mechanisms acquired from *P. aeruginosa* during the chronically infection of CF airway. This is due by a peculiarly and unusually property of *P. aeruginosa* found in CF lung: its hypermutable ability (Oliver et al., 2000). This refers to property that permits to react promptly to changes of environment, modifying the gene profile and increasing the frequency of mutation events in the genome, a behavior that is not found in other clinical settings. The conversion in a mucoid phenotype of *P. aeruginosa* permits to gain more resistant to adverse

external changes and highly pro-inflammatory (Fig.1) (Pedersen, 1992). The persistence of *P. aeruginosa* in CF lung is sustained by biofilms formation, which is dependent from a process of QS (Pesci and Iglewski, 1997; Singh et al., 2000). The biofilm protects the microcolonies of bacteria against phagocytosis and prevents the penetration by antibiotics (Fig.1C).

Heterogeneous micropopulations of mutated *P. aeruginosa* have been found in CF lung, in particular, mutations in the transcription factor MexT are associated to: (i) overproduction of the multidrug efflux pump, MexEF-OprN; and (ii) repression of the biosynthesis of quorum-sensing signaling molecule *P. aeruginosa* quinolone signal (Darch et al., 2018; Jorth et al., 2015; Olivares et al., 2012). In support of this, analysis of bronchoalveolar lavage fluid (BALF) obtained from pulmonary lobes of CF patients showed that different lung areas were infected by mucoid/non-mucoid mixed strains (Malhotra et al., 2019). The genomic instability of mucoid *P. aeruginosa* favors the accumulation of new mutations in vivo, determining frequently "phenotype switch" to planktonic and mucoid form (Ciofu et al., 2008; DeVries and Ohman, 1994; Schurr et al., 1994). Continue changes of bacterial phenotype are fundamental to establishing chronic infection, where now the bacteria must survive despite host defense strategies, including repeated cycles of antibiotics. To achieve this, *P. aeruginosa* has also developed immune-evasive strategies, such as exoproducts that prolong the survival in the host, and expressed antibiotic resistance proteins, such as MexEF-OprN, rendering it unrecognizable to immune system and resistant to therapies.

P. aeruginosa releases elastase and alkaline protease to cleave immunoglobulins, complement members and cytokines. Exotoxin A inhibits the phagocytosis while the siderophores, detected in CF sputum, are released to break down the intercellular tight junctions to impair the mucociliary clearance (Haas et al., 1991).

The consolidation of chronic *P. aeruginosa* infection is also due by an inefficient CF cellular and humoral immune defense that fail to clearing bacterial infection mounting an exaggerated inflammatory response (Patergnani et al., 2020). CF neutrophils exhibit: compromised metabolism (McElvaney et al., 2019), altered chlorination (Painter et al., 2006) and excessive release of ROS, DNA, Neutrophil Extracellular Traps and proteases (Clancy et al., 2018; Galli et al., 2012; Keir et al., 2021). CF macrophages fail to polarize into M2 and show reduced selective autophagic activity, becoming a replicative niche for bacteria (Assani et al., 2017; Lamothe and Valvano, 2008; Painter et al., 2006; Ratner and Mueller, 2012; Tarique et al., 2017).

In the 1996 was published the first evidence that airway epithelial cells are capable of ingesting *P. aeruginosa*, a further putative pulmonary defense mechanism that reminiscent the xenophagy (Pier et al., 1996). The authors showed that airway cells expressing CFTR mutant were less capable of ingest the pathogen. Reduced xenophagy in CF airway cells was recently confirmed with higher number of colony-forming unit/mL and with the interactions between xenophagic receptors and invading *P. aeruginosa* that have been observed in CF airway cells compared with non-CF cells, indicating that the reduced bacterial clearance capacity is also intrinsic in CF airways (Rimessi et al., 2020). Thus, the defective xenophagic activity of CF immune and airway cells contribute to persistent exacerbation of inflammation, which unable to sequester and destroy the intracellular pathogens, these surviving and persisting in the CF lungs (Cabrini et al., 2022). The clinical state of CF patients worsens when chronic *P. aeruginosa* infection becomes established. In fact, screened infants with chronic *P. aeruginosa* infection showed a more rapid decline in chest X-ray scores than those uninfected (Kosorok et al., 2001), correlating with an increase of mortality (Durda-Masny et al., 2021).

4. Effects of *P. aeruginosa* on mitochondrial functions in cystic fibrosis

In CF, the excessive inflammation due to chronic infection by *P. aeruginosa* plays a critical role in the progression of disease. The impact of this pathogen, together with consequences of defective CFTR channel, affects the mitochondria, organelles that have gained much attention due to their involvement in several inflammatory-related diseases. Exogenous or endogenous stimuli that affect mitochondrial activity are sufficient to activate innate immune responses (Martino et al., 2009), underlying that cells use the mitochondrial stress to potentiate immunity when alterations of homeostasis occur (Pellegrino et al., 2014). Typically, mitochondrial stress responses and quality control pathways intervene for the preservation of mitochondrial functions under stress, avoiding the exacerbation of inflammatory responses (Patergnani et al., 2020). In CF, the recurrent infections cause a chronic mitochondrial stress that leads to a persistent mitochondrial dysfunction, which it is not fully rectified by the intervening of mitochondrial stress responses. This lacked recovery of mitochondrial homeostasis in airway cells is crucial for the pathogenesis of CF lung disease and for the exacerbation of the inflammatory responses.

Mitochondria are the sources of mtDAMPs (ROS, mtDNA, Cardiolipin) that are released into the cytosol or in the extracellular milieu, where, recognized by Toll-like receptors (TLRs) or cytosolic NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs) trigger the formation of a multi-protein complex, known as "inflammasome" that, in turn, promotes the pro-inflammatory cytokines release (Broz and Dixit, 2016). A detailed role for mitochondria in NLR activation has been proved for NLR family pyrin domain containing 3 (NLRP3) and NLR family CARD domain containing 4 (NLRC4/IPAF) (Sorbara and Girardin, 2011). The activation of inflammasome requires two steps: (i) "priming": pro-inflammatory stimuli induce the expression of various inflammasome members mediating the NF-κB-dependent transcriptional pathway (Liu et al., 2018); (ii) "activation": requires physical interactions with mtDAMPs for the assembly of inflammasome and the consequent auto-cleavage of pro-caspase-1, which produces the mature cytokines IL-1β and IL-18 (Liu et al., 2018). The migration of inflammasome to mitochondria is crucial for the "activation step" and it is due by mitochondrial antiviral signaling pathway (MAVS) and mitochondrial-anchored protein ligase (MAPL) binding (Park et al., 2013; Subramanian et al., 2013). Furthermore, the two steps required for NLRP3 inflammasome activation are linked to the new synthesis of mtDNA, underlying the role of mitochondria in both steps involved (Zhong et al., 2018). In addition, mtDNA may be oxidized by mitochondrial ROS, and the resulted oxidized mtDNA also activates NLRC4/IPAF inflammasome, that is known to be stimulated by *P. aeruginosa* (Jabir et al., 2015; Sutterwala et al., 2007; Tolle et al., 2015).

In CF airway cells, it has been demonstrated that during *P. aeruginosa* infection NLRP3 redistributed to the mitochondria (Rimessi et al., 2015). The mitochondrial translocation in these cells was associated with the deubiquitination of NLRP3 inflammasome, an event required for its activation (Juliana et al., 2012; Rimessi et al., 2015). Upon *P. aeruginosa* infection, CF airway cells showed also a marked mitochondrial dysfunction respect to healthy cells, characterize by reduced mitochondrial membrane potential, increased ROS production and mitochondrial network fragmentation (Fig.2). However, one of the constituents of *P. aeruginosa*, flagellin, played an important role in this dysfunction (Rimessi et al., 2015): the non-motile *P. aeruginosa* mutant was not able to alter mitochondrial potential and morphology in CF airway cells and, at the same time, its inability to induce mitochondrial ROS production and IL-1β and IL-18 release, highlighting the role of flagellin to promote mitochondrial dysfunction

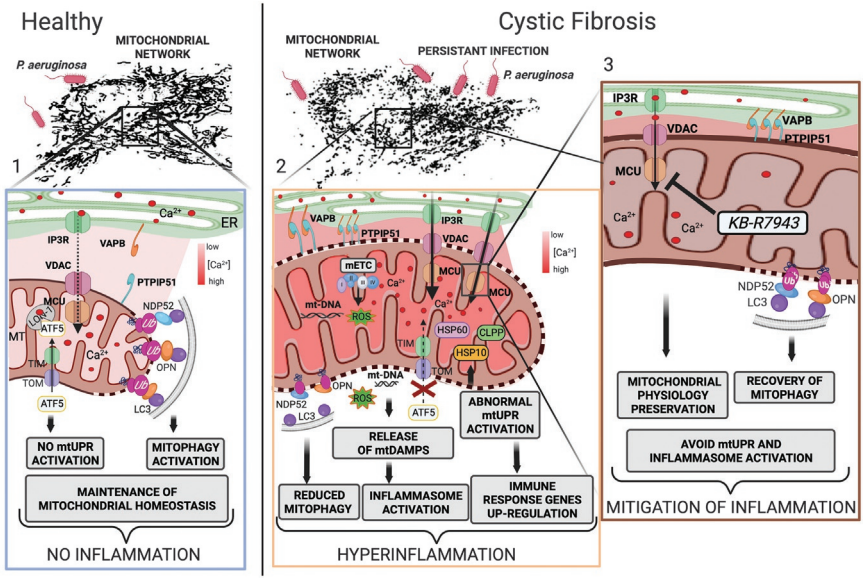

Fig. 2 The role of mitochondria during pathogen infection in CF airways. (1) In healthy condition, mitochondrial stress responses are triggered to restore the mitochondrial homeostasis during *P. aeruginosa* infection. The UPR^{mt} is regulated by the transcription factor ATF5, which in is imported into mitochondria to be degraded by LON-1. Damaged mitochondrial portions are removed by mitophagy, where signaling pathways promotes ubiquitination of OMM proteins while the mitophagic receptors optineurin (OPN) and NDP52, act as adaptors to recruit autophagosomal membranes to mitochondria, interacting with LC3. (2) In CF airway epithelial cells, persistent *P. aeruginosa* infection causes mitochondrial disfunctions, such as network fragmentation. The abnormal ER-mitochondria Ca^{2+} transfer, due by increased inter-organelle crosstalk mediated by VAPB-PTPIP51 tethering, induces the reduction of mitophagy. In turn, abnormal UPR^{mt} activation is characterized by nuclear translocation of ATF5 and expression of HSP60, HSP10 and CLPP. The main consequences of these events, together with the release of mtDAMPs (like ROS and mtDNA), promote exacerbation of inflammatory environment through inflammasomes activation. (3) Pharmacologically inhibition of mitochondrial Ca^{2+}-uptake, by MCU inhibitor KB-R7943, prevents *P. aeruginosa*-triggered mitochondrial dysfunctions in CF airway cells leading to mitigation of pulmonary inflammation through the rescue of mitophagic activity and reduced inflammasomes activation.

and inflammasome activation. Indeed, Rimessi et al. through the modulation of single inflammasome members (including NLRP3, NLRC4 and casp-1) showed that NLRP3 and NLRC4 inflammasomes cooperate leading to worsen inflammatory responses in CF airway cells during *P. aeruginosa* infection (Rimessi et al., 2015). The activation of inflammasomes is corroborated by the high levels of IL-1β that have been measured in BALF,

sputum, serum, airway epithelial and monocyte cells from CF patients (Bonfield et al., 1995; Douglas et al., 2009; Iannitti et al., 2016; Levy et al., 2009; Rimessi et al., 2015; Scambler et al., 2019). The IL-1β release contributes further to alter the mitochondrial potential, inducing mitochondrial ROS production, NF-κB activation and thus the generation of a loop that exacerbate the inflammatory state of CF lung (Escames et al., 2012; Lopez-Armada et al., 2006).

The higher susceptibility to *P. aeruginosa*-dependent mitochondrial dysfunction in CF airway cells respect to healthy is due by mitochondrial Ca^{2+}-overload measured in CF cells as consequence of defective CFTR channel (Rimessi et al., 2015). Mutations in CFTR gene are associated to abnormal intracellular Ca^{2+} concentration that has been observed in several human CF patient-derived cells, including airway and immune cells (Banschbach et al., 1978; Rimessi et al., 2015, 2021; Robledo-Avila et al., 2018; Waller et al., 1984). Result of an enhanced Ca^{2+}-entry mediated by plasma membrane Ca^{2+} channels and by reduced Ca^{2+}-efflux operated by Ca^{2+}-pumps, which it favors a major ER-mitochondria Ca^{2+} transfer during *P. aeruginosa* infection, with heavy repercussions on cell functions and inflammatory responses (as reviewed in (Rimessi et al., 2021)).

During pathogen infection, the interactions between ER and mitochondria change to support a variety of physiological processes. This structural perturbation represents a physiological response of the cell to the alteration of mitochondrial bioenergetics under stress stimuli (Bonora et al., 2012). In CF airway cells, the ER-mitochondria coupling resulted increased during *P. aeruginosa* infection respect to healthy cells (Rimessi et al., 2020). *P. aeruginosa* induced an increased expression of ER-resident vesicle-associated membrane protein-associated protein B (VAPB) and of OMM-resident tyrosine phosphatase interacting protein 51 (PTPIP51) tethers in CF airway cells, that was associated to higher percentage of VAPB-PTPI51 co-localization, which potentiated the inter-organelle Ca^{2+} transfer via MCU complex (Fig. 2) (Rimessi et al., 2020). Controlling the mitochondrial Ca^{2+}-uptake in CF airway cells, through genetic manipulation or pharmacological inhibition of MCU, prevented *P. aeruginosa*-triggered mitochondrial dysfunction (Fig.2) (Rimessi et al., 2020). During *P. aeruginosa* infection, the MCU-silenced or KB-R7943-pretreated CF airway cells presented lower mitochondrial ROS production, inflammasomes activation and IL-1β and IL-18 release, highlighting that the mitochondrial Ca^{2+} signal plays a crucial role in the inflammatory responses in CF lung disease. Furthermore, decreasing the mitochondrial Ca^{2+}-overload limited the VAPB-PTPIP51 coupling,

indicating that the enhanced ER-mitochondria interactions is a compensatory response to endorse ER-mitochondria Ca^{2+} transfer in CF cells during *P. aeruginosa* infection.

The enhanced VAPB-PTPI51 coupling and thus the increased ER-mitochondrial Ca^{2+} transfer, induced by *P. aeruginosa* infection in CF airway cells, led also to downregulate selective autophagic responses, such as mitophagy and xenophagy (Fig. 2) (Rimessi et al., 2020). Under stress conditions, PTEN-induced putative protein kinase 1 (PINK1) accumulates on the OMM of damaged mitochondria, where phosphorylating ubiquitin (Ub) promotes the mitochondrial translocation of E3 ubiquitin ligase Parkin. This last stimulates the ubiquitination of several OMM resident proteins, including mitofusin (MFN) and voltage-dependent anion-selective channel (VDAC) protein, defining the signal for the enrollment of Ub-binding mitophagic receptors, such as calcium-binding and coiled-coil domain 2 (NDP52) and optineurin (OPTN), useful to engulf the damaged mitochondria by the microtubule-associated protein 1-light chain 3 (LC3)-positive autophagosome (Fig.2) (Geisler et al., 2010; Pickles et al., 2018).

P. aeruginosa triggers mitophagy in airway epithelial cells, leading to the recruitment of Parkin, NDP52, OPTN and LC3-II to damaged mitochondria. In CF, the lower mitochondrial distribution of Parkin and mitophagic receptors was associated to a reduced kinetic of mitochondrial sequestration into LC3-positive autophagosome during pathogen infection, which, in turn, led to accumulate damaged mitochondria that contributed to exacerbate the inflammation in CF airway cells (Rimessi et al., 2020). Treatments with KB-R7943 attenuated the inflammation in CF lung also rectifying the mitochondrial clearance capacity, a fundamental process useful to preserve the mitochondrial homeostasis in CF airway cells upon *P. aeruginosa* infection (Rimessi et al., 2020).

The persistent presence of altered mitochondria in CF airway cells during *P. aeruginosa* infection activated the transcriptional stress response, UPR^{mt}, in order to recover the mitochondrial homeostasis (Rimessi et al., 2020). Major nuclear translocation of ATF5 and an increased expression levels of UPR^{mt} reporters, such as heat shock protein (HSP) 10, HSP60 and caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP) was showed in CF airway cells during *P. aeruginosa* infection (Fig.2). The mitochondrial clearance capacity of CF airway cells is further compromised by the abnormal UPR^{mt} activation, which favors the expression of master negative-regulator of autophagy, mTOR, through the ATF5-dependent transcriptional program (Sheng et al., 2011). The overexpression of ATF5

determined a further reduction of autophagy in CF airway cells during *P. aeruginosa* infection, this was related to an augmented sensitivity to infection as revealed by increased NLRP3 inflammasome-dependent IL-18 and IL-1β release (Rimessi et al., 2020). The persistence of UPR^{mt} activation affects thus the pathogenesis of CF lung disease, worsening the mitochondrial clearance capacity and the inflammatory responses of CF airway cells during pathogen infection.

All these findings show how the mitochondria and the mitochondrial Ca^{2+} signaling play a pivotal role in *P. aeruginosa*-triggering pulmonary inflammation in CF. The regulation of mitochondrial Ca^{2+} uptake is thus crucial to mitigate mitochondrial dysfunction and the inflammatory state of CF lung, controlling inflammasomes and UPR^{mt} activation, while the selective autophagic responses are rectified.

5. Conclusion

The mitochondria are fundamental for maintaining the integrity and health of the whole organism, in charge to the cells provide energy, maintain Ca^{2+} homeostasis, regulate redox and innate immune reactions, and take part in apoptosis. Their dysfunction contributes to aggravate the pathogenesis of different inflammatory-related diseases, including CF, worsening the patient's clinical outcome. Their origin and their similarity to bacteria could suggest that these organelles can suddenly become a threat. Evolved from ancient endosymbiotic proteobacteria, the mitochondria share conserved structural motifs with them: (i) circular DNA; (ii) similar double-layered membrane and arrangement, like cristae and mesosome; (iii) similar ribosomes and ETC proteins; (iv) both are formed by a similar process of binary fission (Boguszewska et al., 2020).

These similarities suggest that mtDNA or proteins may act as mtDAMPs, triggering similar signaling pathways activated by bacterial PAMPs increasing the severity of inflammatory responses, and thus exacerbating the pathological condition, as demonstrated in CF. Thus, in CF will be of crucial importance for airway and immune cells that the integrity of mitochondrial physiology will be maintained and protected overtime through the activation and the restoration of mitochondrial stress responses, even during a bacterial infection. A new aspect associated to mitochondrial dysfunction in CF pathogenesis is the pathological role of senescence. Mitochondrial alterations, such as depletion of mtDNA or inhibition of the electron transport chain, may induce mitochondrial dysfunction associated senescence

(MiDAS) (Wiley et al., 2016). A serine-threonine kinase mTOR-dependent cellular growth arrest related to secretory phenotype, which the prolonged cellular arrest promotes the secretion of several molecules with paracrine effects, including cytokines, proteases, growth and angiogenesis factors, that influence the pro-inflammatory microenvironment and compromise tissue structure and function (Correia-Melo et al., 2016; Hernandez-Segura et al., 2018). Particularly, mTOR activity has been found to be upregulated in CF airway epithelial cells, and its activity controls CFTR stability and expression, suggesting that also MiDAS may be implicated in CF (Bezzerri et al., 2019; Reilly et al., 2017).

In the future, it will be useful: (i) to understand whether the amount of released mtDAMPs determines the severity of clinical outcome and the degree of mortality; (ii) to study whether the presence of circulating mtDAMPs may be used as prognostic marker to predict the severity of inflammatory-related diseases; (iii) to investigate possible additional mtDAMPs able to trigger an immune response.

A hallmark of CF lung disease is the early and persistent *P. aeruginosa* infection, characterized by severe and sustained inflammation (Cabrini et al., 2022). In healthy host, pathogens are removed from lungs through mechanical (mucocilary clearance) and immunological strategies without triggering the inflammatory response. In CF, mechanical and immune defenses are inefficient in clearing bacterial infections, mounting an "exaggerated" inflammatory response which contribute to bronchial tissue damage and progressive respiratory insufficiency. This suggests that the basal CFTR defect is a pro-inflammatory condition itself, which: (i) predisposes a microenvironment favorable for bacterial growth and expansion (due by the mucus abnormality and the impaired mucociliary clearance); (ii) renders the mitochondria more susceptible to pathogen-triggering dysfunction with consequent activation of mitoinflammation (due to mitochondrial Ca^{2+} signaling and mitochondrial stress responses impairment (Patergnani et al., 2020; Rimessi et al., 2021)) and senescence; (iii) weakens cellular and humoral immune defenses that fail to clear bacterial infection (defective phagocytes killing and immune cells imbalance). Initially, the pro-inflammatory condition related to basal CFTR defect is silent, in fact, no excessive inflammatory cytokine expression and leucocytes infiltration has been observed in absence of bacterial infection in CF patient, while is full blown once the pathogen infection has been acquired (Armstrong et al., 1995). The pathological condition is thus exasperated by superb ability of *P. aeruginosa* that use its endogenous toxins and strategies to exploit host intracellular signaling to provide a supportive local niche to facilitate the

growth and the survival to the stressful host environment characterized by host defenses and persistence of antibiotics. Indeed, the evolution of *P. aeruginosa* during chronic infective/inflammatory condition has implication not only on the inherent multidrug resistance ability of pathogen but also on the efficiency of new CFTR modulators that intervene to rescue the function of CFTR protein (as reviewed in Cabrini et al., 2022), suggesting that of new and alternative anti-inflammatory and anti-bacterial strategies are desperately needed in CF.

Conflicts of interest

The authors declare that there are no competing interests in relation to this work.

Author contributions

All authors contributed substantially to discussions of the content. All authors contributed to writing the article and to reviewing and/or editing the manuscript before submission.

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