Chapter 13

Autophagy in Cystic Fibrosis Pathogenesis and Treatment

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Additional information is available at the end of the chapter

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Abstract

Cystic fibrosis (CF) is a fatal, genetic disorder that critically affects the lungs and is directly caused by mutations in the *CF transmembrane conductance regulator* (*CFTR*) gene, resulting in defective CFTR function. In epithelial cells, the CFTR channel conducts anions and plays a critical role in regulating the volume and composition of airway surface liquid. This thin layer of aqueous fluid and mucus covering the airway surface facilitates mucociliary clearance, bacterial killing, and epithelial cell homeostasis. The importance of the CFTR channel in macrophages was revealed in recent work that demonstrated that defective CFTR function is accompanied by impaired innate immune responses to specific infections. Notably, most CF-associated infections are caused by microbes that are cleared by autophagy in healthy cells. Autophagy is a highly regulated biological process that provides energy during periods of stress and starvation. Autophagy clears pathogens, inflammatory molecules, and dysfunctional protein aggregates within macrophages. However, this process is impaired in CF patients and CF mice, as their cells exhibit limited autophagy activity. The mechanisms linking a malfunctioning ion channel function to the defective autophagy remains unclear. In this chapter, we describe and discuss the recent findings indicating the presence of several mechanisms leading to defective autophagy in CF cells. Thus, these novel data advance our understanding of mechanisms underlying the pathobiology of CF and provide a new therapeutic platform for restoring CFTR function and autophagy in patients with CF.

Keywords: cystic fibrosis (CF), autophagy, Rab GTPases (Rabs), CF-associated bacteria, autophagy therapeutics

1. Introduction

Cystic fibrosis (CF) is the most common life-threatening genetic disease in North America and Europe. The birth prevalence of CF is estimated to be one in 3500–4500, with 200–300 new cases each year in Europe. The typical form of CF is diagnosed during early childhood and is characterized by recurrent pulmonary infections, pancreatic insufficiency, and elevated chloride concentrations in sweat. CF is a multi-organ disorder, however infections often occur in the lungs accompanied with severe inflammation and tissue destruction [1]. CF is directly caused by mutations in the *CF transmembrane conductance regulator* (*CFTR*) gene, resulting in defective CFTR function. Over 1400 mutations have been identified in the CFTR gene, the most common mutation leading to CF is deletion of phenylalanine at position 508 (F508del) and is present in over 70% of the CF alleles [2, 3]. Most of the work discussed in this chapter refers to the F508del-CFTR.

The previous commonly accepted hypothesis for CF pathology is the excessive secretion of thick mucus that remains in the lungs and is accompanied by impaired mucociliary clearance. This viscous mucus layer predisposes CF patients to chronic pulmonary infections. An intriguing speculation arose from the specificity of organisms that tend to infect CF patients. We hypothesized that the susceptibility of CF patients to these infectious agents is due to weak autophagic activity since most of the organisms that tend to cause chronic infection in CF are controlled by autophagy in healthy cells [4–6]. Autophagy is a highly regulated biological process that provides energy during periods of stress and starvation [7] and is typically induced upon glucose or amino acid starvation. Autophagy clears pathogens, inflammatory molecules, and dysfunctional protein aggregates within macrophages. Autophagy proceeds through sequential steps that begin with the formation of the phagophore or isolation membrane at a pre-autophagosomal structure (discussed in chapter of this book) [8]. The nascent autophagic membrane elongates to form a double-membrane autophagosomes that captures regions of cytoplasm, damaged mitochondria, or aggregated proteins. Upon maturation, the autophagosome containing the isolated cargo then fuses with the lysosome to form a single membrane compartment called the autolysosome. The autophagosomal cargo is then degraded in this compartment by lysosomal acid hydrolases and other degradative enzymes. The resulting degradation products including free amino acids, fatty acids, and nucleotides are released to the cytoplasm by the action of lysosomal permeases, where they may be reutilized for anabolic pathways [9, 10]. However, this process is impaired in CF patients and CF mice, as their macrophages and epithelial cells exhibit limited autophagic activity. The mechanisms linking a malfunctioning ion channel to the defective autophagy remains unclear.

Until recently, it was believed that the production of thick mucus and the impairment of mucociliary clearance was the main underlying culprit, allowing the persistence of specific infections in the CF lung. The idea of the existence of an innate immune deficiency disorder in CF was not examined until lately. The discovery that the innate immune functions of macrophages and neutrophils are disrupted in CF was a turning point in the CF field and in understanding the pathobiology of CF. Several years were consumed to provide what is now undisputable data confirming that CF should be considered an innate immune disorder.

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In this chapter, we describe and discuss the recent findings in the field, which demonstrate that CF is a newly recognized innate immune deficiency disorder. We will discuss several reports demonstrating that macrophage functions are disrupted in CF contributing to the pathobiology of the disease. This chapter, encompassing recent data in CF, suggests that targeting autophagy may be exploited as a novel strategy for treatment of CF.

2. Cystic fibrosis

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is an integral membrane glycoprotein that functions as a cAMP-activated and phosphorylation-regulated Cl channel at the apical membrane of epithelial cells. CFTR is a member of the ATP-binding cassette transporter superfamily. It is a multi-domain glycoprotein whose biosynthesis, maturation, and functions involve multi-level posttranslational modifications and complex folding processes to reach its native, tertiary conformation. The topology of CFTR includes two transmembrane-spanning domains, two nucleotide-binding domains, and a regulatory domain, which is a unique feature among ATP-binding cassette transporters (**Figure 1**) [11]. The newly synthesized CFTR emerges out of the ribosome and is targeted through the signal recognition particle to the ER membrane translocon [12, 13]. The CFTR polypeptide chain emerges into the ER lumen, and its glycosylated helping stabilize the protein. CFTR follows the secretory pathway through the Golgi in order to reach the plasma membrane [14]. The recycling of internalized CFTR channels is important for maintaining a functional pool of CFTR at the plasma membrane.

Only 20–40% of the nascent chains achieve folded conformation, whereas the remaining molecules are targeted for degradation by endoplasmic reticulum, lysosomes, or autophagy. A large number of mutations impair processing of CFTR. Growing knowledge of CFTR biosynthesis has enabled understanding of the cellular basis of CF and has brought to light various potential targets for novel and promising therapies [15]. The most common mutation leading to CF is deletion of phenylalanine at position 508 (F508del) and is present in over 70% of the CF alleles [2, 3]. As mutant CFTR is targeted for degradation by the proteasome, the formation of protein aggregates occurs, hence provoking an unfolded protein response (UPR) [16]. Other common mutations such as the G551D exhibit defective CFTR function, however it does not aggregate or elicit a UPR. Comparing the phenotypes and immune functions of the F508del and G551D mutants will discern if defective bacterial clearance and uncontrolled inflammation are due to defective channel function, UPR, or both.

Hyper-inflammation and failure to clear infection is recognized as a leading cause of lung tissue destruction in CF [17] that can be explained, at least in part, by incompetent autophagy machinery in cells with a dysfunctional CFTR channel [18]. Bronchoalveolar lavages from CF patients contain high levels of the pro-inflammatory cytokine interleukin (IL)-1β [1, 19–26]. IL-1β is primarily expressed as a precursor inactive molecule that is later cleaved by caspase-1 to yield active 17-kDa IL-1β [27]. The biological activities of IL-1β include promoting inflammatory responses and leukocyte infiltration. Autophagy directly regulates the level of pro-

IL-1β in response to lipopolysaccharide (LPS) and infections [28, 29]. Interestingly, stimulation of autophagy by rapamycin dramatically reduced signs of inflammation in the murine CF lung [30, 31].

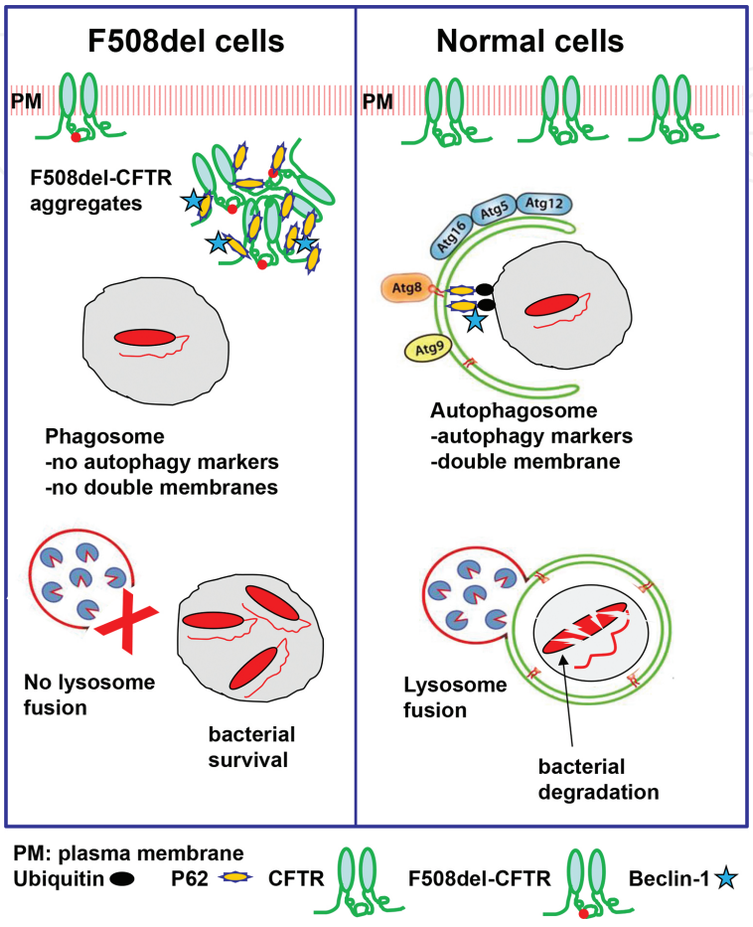

Figure 1. Autophagy process is impaired in CF cells preventing bacterial clearance.

Several reports demonstrated multiple malfunctions in adaptive and innate immune responses in CF. Lack of functional CFTR in CD3+ lymphocytes leads to aberrant cytokine secretion and hyper-inflammatory adaptive immune responses [32] while producing high levels of IL-1β [1, 19]. Naive cystic fibrosis T cells are intrinsically predisposed to differentiate toward a Th17 phenotype [33]. Therefore, CF is a multifaceted immune deficiency disease.

3. Rabs and the cytoskeleton: common modulators or innocent bystanders for autophagy and CFTR trafficking?

Rab (Ras-related proteins in brain) proteins are key regulators of both vesicular transport and trafficking of proteins [34]. Several Rab GTPases have been implicated in the regulation of the

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intracellular transport and the plasma membrane delivery of CFTR. The trafficking of CFTR from the plasma membrane to early endosomes is controlled by RAB5 [35, 36]. RAB7 regulates the movement of CFTR away from the recycling pathway and into late endosomes and also from late endosomes to lysosomes for degradation [37]. RAB9, however, can move CFTR away from lysosomal degradation by mediating its transport from late endosomes back to the trans-Golgi, from which CFTR may reenter the secretory pathway leading to plasma membrane insertion [37].

Growing knowledge of CFTR biosynthesis has enabled understanding of the cellular basis of CF and has brought to light various potential targets for novel and promising therapies [15]. Although some *in vitro* studies have shown that F508del-CFTR cell surface expression can be increased through the manipulation of key Rab GTPases, the mechanisms involved are still unclear. Rabs are also related to autophagy by regulating the transport and fusion of autophagosomes. However, it remains unclear how each cycle of Rab activation/inactivation is finely regulated. There is evidence indicating that RAB1, RAB5, and RAB7 participate in certain steps of autophagosome development and maturation [38], but the specific function of some of these Rab proteins remains poorly characterized. Conversely, RAB7, a low molecular weight GTPase found mainly on late endosomes, has been extensively studied [38]. By interacting with its partners (including upstream regulators and downstream effectors), RAB7 regulates mechanisms in endosomal sorting, biogenesis of lysosome, and phagocytosis [37]. Particularly, RAB7 governs early-to-late endosomal maturation, microtubule minus-end as well as plus-end directed endosomal migration, and endosome-lysosome transport through different protein–protein interaction cascades [34, 39]. In addition, RAB7 directs the maturation of autophagosomes, by guiding the trafficking of cargos along microtubules to participate in the fusion step with lysosomes [38]. Notably, activation of Rab7 is impaired by bacteria that tend to infect CF patients such as *Burkholderia cenocepacia*, accounting at least in part for the inability of the vacuole to merge with lysosomes [40]. *Staphylococcus aureus* also modulates Rabs to establish infection [41]. Whether specific nonfunctional Rabs promote aberrant CFTR trafficking and autophagy malfunction through common mechanisms in CF remains to be elucidated.

4. "Eat-me" signaling molecules: Are they trapped in CFTR aggregates?

The major molecular regulator of autophagy in response to starvation or energy depletion include the mammalian target of rapamycin complex 1 (mTORC1). Inhibition of mTORC1 by starvation or rapamycin results in the activation of autophagy and the start of autophagosome formation. The capacity of autophagy to clear intracellular pathogens such as bacteria, viruses, and parasites is collectively referred to as xenophagy [5], whereas the selective autophagic degradation of mitochondria is denoted as mitophagy and that of protein aggregates is termed aggrephagy. But how do autophagosomes find their targets? The modification of targets by ubiquitination represents a signal for selection of substrates to the autophagy pathway.

Mammalian cells ubiquitinate bacteria that erroneously enter the cytosol or their containing vacuole and target them for destruction by autophagy. Adaptors, including p62/SQSTM1 [42],

optineurin (OPTN) [43], NBR1 (neighbor of BRCA1 gene 1) [44–46], and NDP52 (nuclear dot protein 52 kDa) [47], mediate "eat-me" functions by promoting autophagic sequestration of cargo. The adaptor molecule p62 is a ubiquitously expressed cellular protein and its quantity is critical for cell viability [48]. p62 has multiple protein-protein interaction domains, including the ubiquitin-associated domain for binding of ubiquitinated cargo and a LC3 interaction region for binding Atg8/LC3 [49]. p62 plays a role in amino acid sensing and the oxidative Stress response, in addition to its function as an autophagy receptor for ubiquitinated cargos [50]. Most p62 protein in the healthy cell is distributed in the cytoplasm. In response to various stressors though, it is translocated to autophagy substrates such as protein aggregates, damaged mitochondria, and intracellular bacteria [42]. Then, through its LC3-binding domain, p62 engages autophagosomes.

Autophagy is responsible for the degradation of p62. Therefore, impairment of autophagy is usually accompanied by massive accumulation of p62 followed by the formation of aggregate structures positive for p62 and ubiquitin [50]. This accrual is a defining characteristic of impaired autophagy. Aggregation occurs due to both the predilection for self-oligomerization and the ubiquitin-binding capabilities of p62 [51]. Notably, p62 accumulates in CF macrophages and promotes the sequestration of mutant CFTR (**Figure 1**). These aggregates, in turn, consume important autophagosome-needed proteins, such as BECN1 and LC3 [30, 31]. Recent reports demonstrate that the adapter protein NDP52 directly binds to ubiquitinated bacteria and facilitates the assembly of an autophagic membrane that surrounds these invaders [47]. Interestingly, NDP52 can also bind ubiquitinated bacteria-containing vacuole when p62 is drastically reduced [30, 31]. Optineurin can mediate the removal of protein aggregates through an ubiquitin-independent mechanism. In addition, this protein can induce autophagy upon overexpression or mutation [43]. NBR1 and p62 cooperate in the sequestration of misfolded and ubiquitinated proteins in p62 bodies and are both required for their degradation by autophagy. Recently, NBR1 was found to be necessary and sufficient for pexophagy [44–46]. Whether NBR1, optineurin, and NDP52 play important roles in CF-associated autophagy is still unknown.

5. Function of CFTR channel in epithelial cells

CFTR is an anion channel permeable to chloride and bicarbonate [2, 52]. Upon activation, CFTR transports chloride following its electrochemical gradient. In the lung, CFTR is expressed at the apical membrane of bronchial cells where it regulates chloride transport and fluid homeostasis [53]. The absence of functional CFTR in the lung results in abnormal surface hydration and decreased airway surface fluid. Thus, mutations in the CFTR protein results in the accumulation of thick mucus at the surface of epithelial cells, leading to impairment of pathogen clearance and dysregulated inflammatory responses that in turn results in chronic infection and inflammation [54]. In addition, epithelial cells expressing mutant CFTR exhibit weak autophagy activity.

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6. Function of CFTR channel in macrophages

Macrophages are central innate immune cells that engulf invaders within a vacuole and target them to fuse with the lysosome for degradation. Therefore, lysosomes contribute to antimicrobial capacities by fusing with the pathogen-containing, intracellular vacuole [55]. Lysosomes are acidic compartments filled with various acid hydrolases, NADPH oxidases, and oxygen radicals that degrade and break down proteins, lipids, and polysaccharides.

The lung disease seen in CF was accordingly suggested to result in part from lysosomal dysfunction [56], yet the exact location and function of CFTR in macrophages are still debatable. A report showed impaired bacterial killing due to impaired function of antibacterial proteins at low pH in a CF pig model [57]. Another study demonstrated that acidification requires anion transport through CFTR [58]. More recently, defective lysosomal acidification was also invoked as the mechanism underlying CF lysosome malfunction [59]. Others failed to confirm the involvement of CFTR in acidification [60, 61]. Both these reports are plausible. When macrophages are infected with autophagosome-resident organisms such as *B. cenocepacia*, fusion with the lysosome is impaired and acidification is reduced [30, 31, 56]. Yet, when they are infected with phagosome-resident organism such as *Escherichia coli*, fusion with the lysosome moves swiftly and the bacterium is degraded within minutes in an acidified compartment [30, 31]. These results suggest that the defect in lysosomal degradation is only associated with autophagy. This explains the prevalence of several autophagy-related organisms in CF. Actually, an acceptable approach would be to examine organisms that tend to infect CF patient and test whether they are cleared by autophagosomes in healthy cells as was achieved for *B. cenocepacia* and *Pseudomonas aeruginosa*. However, on the other hand, not every organism that interacts with autophagosomes survives in CF macrophages. For example, in our hands, *Legionella pneumophila* that is cleared by autophagy in wild-type healthy macrophages and survives in autophagosomes in permissive macrophages fail to establish infection in CF macrophages (unpublished observation). How would a chloride channel alter autophagic activity is still unclear. Taken together, the role of CFTR in lysosomal function still awaits more conclusive reports.

7. CFTR in neutrophils

Polymorphonuclear neutrophils (PMNs, neutrophils) are responsible for the earliest innate immune response to infection and most of their antimicrobial activity against ingested microbes is confined within phagosomes. However, exuberant neutrophil activation can culminate in extracellular release of oxidants and granule contents that leads to local damage to healthy tissue. Neutrophils function to kill microbes through compartmentalization by use of membrane-bound phagosomes, where toxic oxidants such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are generated [62]. The azurophilic granule protein myeloperoxidase (MPO) catalyzes the oxidation of Cl^- to form HOCl. Neutrophils predominate in the CF patient's lung and is a major contributor to the inflammation and destruction of the lung [63].

Yet, despite a successful inflammatory response, neutrophils fail to eradicate invading microbes in the CF lung [64]. CFTR dysfunction results in impaired intraphagosomal HOCl production and neutrophil-mediated microbial killing. Notably, the *Cftr* $^{-/-}$ lungs are deficient in bacterial clearance, despite the sustained neutrophilic infiltration and accompanying inflammation [65]. These events demonstrate that neutrophils with nonfunctional CFTR have reduced capability to clear infection, yet remain capable of releasing inflammatory molecules that destroy the lung tissue leading to decline in lung function. Some studies suggested that CFTR channel expression in neutrophils and its dysfunction can affect neutrophil chlorination of phagocytosed bacteria [65] and that CFTR-dependent chloride anion transport contributes significantly to *P. aeruginosa* killing by normal neutrophils [63]. Others have reported that neutrophils from mice expressing the F508del-CFTR or mice lacking CFTR in myeloid cells have a pro-inflammatory phenotype after lipopolysaccharide or bacterial [64] challenge, thus suggesting again that CFTR expression in neutrophils might regulate their function [66].

In the CF lung, there is overproduction of the neutrophil chemotactic cytokine interleukin 8 (IL-8). This leads to excessive infiltration of neutrophils [67]. Lung infections cause significant morbidity and mortality in patients with CF, even in the presence of neutrophil infiltration into infected lungs. Thus, disturbance in innate neutrophil function in CF includes excessive recruitment [65], hyper-production of oxidants, and increased release of degradative enzymes [64]. Thus, it is evident that CF is indeed an innate immune disorder involving several malfunctioning immune cells.

8. Autophagy and cystic fibrosis

Several autophagy proteins are scarcely expressed in CF cells, yet the underlying mechanism is undefined [30, 31]. This strongly suggests the presence of an epigenic regulation that targets autophagy mRNA in cells bearing mutant F508del-CFTR. Considering the strong implications of microRNAs (miRs) in autophagy [28, 29, 68–70] and given the increasing evidence showing reduced expression of essential autophagy proteins in CF cells, we performed an *in silico* approach to recognize miRs that target autophagy and are highly expressed in CF cells [71]. miRs are evolutionarily conserved class of small (~21–24 nucleotides) noncoding RNAs that play key roles in the transcriptional and posttranscriptional regulation of gene expression [72]. We identified the *Mir17~92* cluster as being deregulated in CF [71]. This cluster generates a single polycistronic transcript that yields six mature *Mirs*: *Mir17*, *Mir18a*, *Mir19a*, *Mir20a*, *Mir19b*, and *Mir92* [73]. miRs can regulate individual stages of these processes [72]. The polycistronic *Mir17~92* cluster was initially linked to tumorigenesis. Whether the elevated levels in CF patients will promote cancer in aging CF population remains to be observed [73–80]. Several specific miRs comprising the *Mir17~92* cluster are overexpressed in CF human and murine macrophages. Their expression is indirectly proportional to the expression of their predicted autophagy-targeted genes. Notably, reducing the inherently elevated expression of *Mir17* and *Mir20a* improves ATG7 and ATG16 expression both *in vitro* and *in vivo* [71]. In addition, reducing *Mir17* and *Mir20a* expression improves CFTR function by restoring

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autophagy expression [71]. Whether other epigenetic regulatory elements contribute to low expression of autophagy proteins is still unclear.

In airway epithelial cells, absence of functional CFTR increases oxidative stress and transglutaminase 2 (TGM2), a calcium-dependent enzyme that creates intra- or intermolecular covalent bonds between proteins. TGM2-mediated cross-linking causes sequestration of BECN1 and its accumulation in histone deacetylase-6 (HDAC6), p62, and ubiquitin-containing cytoplasmic aggresomes. BECN1 sequestration in aggresomes results in the dislodgement of class III PtdIns3K complexes from the endoplasmic reticulum, thereby inhibiting autophagy [30, 81]. In addition, the sequestration of BECN1 within F508-CFTR protein aggregates deprives the cell for an essential factor needed for autophagosome formation.

High levels of p62 promote the aggregation of mutant F508del-CFTR sequestering several autophagy molecules such as BECN1. This p62 buildup could be due to reduced recycling in CF macrophages as a consequence of compromised autophagosome formation and maturation. Notably, p62 downregulation disassembles mutant CFTR and autophagy factors, thus improving autophagy activity and allowing the maturation and trafficking of CFTR to the cell surface in epithelial cells and bacterial clearance in macrophages [30, 31]. Similarly, genetic manipulation or autophagy stimulatory proteostasis regulators such as cystamine restore BECN1 availability and detangle SQSTM1/p62, which partially rescues F508del CFTR function in airway epithelial cells and reduces lung inflammation [82].

9. Cystic fibrosis and infection

There is evidence for autophagy dysregulation in a variety of disease states, including cancer, neurodegenerative diseases, chronic granulomatous diseases, infectious diseases, and autoimmune disorders [83–85]. For this reason, therapeutic modulation of autophagy is of great interest. Autophagy has emerged as a central component of the innate and adaptive immune responses where it plays roles in direct and indirect killing of intracellular and extracellular pathogens, the generation of bactericidal peptides, and antigen presentation [29]. Functions of autophagy that are compromised in CF include bacterial clearance, degradation of protein aggregates, and the elimination of dysfunctional mitochondria. Thus, the restoration of autophagy will have positive therapeutic effects in CF.

Patients with CF are susceptible to Nontypeable *Haemophilus influenzae* (NTHi), *Staphylococcus aureus* (*S. aureus*), *Burkholderia cenocepacia* (*B. cenocepacia*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and nontuberculous *Mycobacterium* (NTM) [86–88]. The major cause of high morbidity and mortality in CF remains the chronic respiratory infections with *P. aeruginosa* [89].

In healthy cells, *P. aeruginosa* is targeted to the autophagy pathway through yet uncharacterized mechanisms [90, 91]. Increased susceptibility of CF cells to *P. aeruginosa* has been attributed to changes in airway liquid composition and enhanced bacterial binding to mucin and epithelial cell receptors such as asialo-GM1 [92]. In addition, disruption of lipid metabolism in CF cells increases innate inflammation in the presence of bacteria. The CFTR protein

may also act as a receptor to *P. aeruginosa* and explains the high infection rate in CF patients [93]. Although largely considered an extracellular pathogen, *P. aeruginosa* can invade host airway epithelial cells where the bacteria can reside for extended periods of time. Pharmacological improvement in autophagy *in vivo* effectively promoted bacterial clearance of *P. aeruginosa* from the lungs.

S. aureus is one of the earliest bacteria detected in infants and children with CF. This pathogen is the single most common CF-associated opportunistic infection, colonizing between 50 and 68% of the population. The rise of methicillin-resistant *S. aureus* (MRSA) in the past 10 years has drawn attention to this organism [94, 95]. Unlike the other common CF-associated pathogens, *S. aureus* escapes from the phagosome upon entering the cell. In healthy cells, cytosolic *S. aureus*, or those contained within damaged phagosomes, are targeted to the autophagy pathway where they inhibit lysosomal fusion [96, 97]. The ability to escape degradation is increased in CF cells [98].

NTM strains infect between 5 and 22% of CF patients and are a growing concern among CF populations due to their increasing prevalence and multi-drug resistance. Infection is often associated with poor clinical outcomes [99]. Although autophagy contributes to clearance of *M. tuberculosis* in healthy cells, it is still unclear whether NTMs can be specifically targeted for degradation by the autophagy machinery [88].

NTHi chronically colonizes the airways of CF patients at a very young age. Recent reports suggest that autophagy may be actively subverted by NTHi by an unknown mechanism.

On the other hand, following phagocytosis, the degradation of *Aspergillus fumigatus* spores requires LC3-associated phagocytosis for effective lysosomal degradation [100]. Whether defective autophagy contributes to the prevalence of this infection in CF is still under investigation.

B. cenocepacia infections are not particularly common in CF patients, afflicting 3–5% of the population, yet they are very difficult to treat due to multi-drug resistance and are associated with a rapid decline in lung function. In healthy macrophages, *B. cenocepacia*-containing vacuoles are targeted to the lysosome for degradation via the autophagy pathway [30, 31]. However, in CF macrophages, *B. cenocepacia* persists in vacuoles that do not acquire LC3 or p62 like their counterparts in wild-type healthy cells. These *B. cenocepacia*-containing vacuoles do not fuse with lysosomes and the bacteria evade degradation (**Figure 1**) [30, 31]. Similar findings were reported in human macrophages derived from CF patients [101]. Together, these data show that correcting autophagy activity in CF will help prevent and eradicate infectious agents that are otherwise detrimental to the CF patients.

10. Targeting autophagy in CF improves CFTR function and bacterial clearance

Many of the opportunistic bacteria that infect the CF lung have employed mechanisms to target phagolysosomal maturation and/or autophagy, suggesting that these are major barriers that

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need to be overcome. Activation of autophagy through rapamycin treatment has shown efficacy in promoting clearance of certain bacteria *in vitro* and *in vivo* [30, 102–104]. Thus, the exploration of alternative autophagy inducing drugs is in the early stages.

Rapamycin was developed as an antifungal agent but its use was abandoned due to the potent immunosuppressive and antiproliferative properties. Recently, it was found that rapamycin inhibits mTOR and therefore induces autophagy [105]. Rapamycin promotes clearance of the CF-associated pathogens *P. aeruginosa* and *B. cenocepacia* both *in vitro* and *in vivo* in mice [30]. This could potentially be beneficial in the treatment of CF-associated lung infection since it is an important cause of decline in lung functions of CF patients [30]. However, rapamycin was most effective when administrated before *B. cenocepacia* infection but not after. The use of immunosuppressive drugs such as rapamycin to treat infections could negatively impact the ability of the patients to fight other opportunistic infections. In addition, rapamycin has been associated with significant lung toxicity in transplant recipients [106–108]. As a result, rapamycin proved the concept that targeting autophagy is needed in CF, but represents a poor candidate for autophagy-inducing therapy in the treatment of CF-associated lung infections, and other more specific candidates are urgently needed. Thus, the field requires a safe autophagy-enhancing approach for CF patients.

Among the most common autophagy-stimulating compounds were the antipsychotic drugs bromperidol, metergoline, thioridazine, and chlorpromazine. However, the psychoactive nature of these compounds and their potentially life-threatening side effects limit their utility, but nevertheless provide a strong theoretical basis for future drug development [109, 110].

Metformin is a drug that activates AMPK and therefore stimulates autophagy via TORC1-dependent and TORC-1-independent methods [111, 112]. Metformin probably has many other mechanisms of action that cannot be explained by the induction of autophagy. Metformin and resveratrol activate SIRT1 that in turn activates autophagy [113, 114].

Certain anticancer drugs have also been found to stimulate autophagy. For example, Perifosine inhibits mTOR signaling through a different mechanism than classical mTOR inhibitors such as rapamycin [115], whereas Tamoxifen, an antagonist of the estrogen receptor, is known to induce autophagy [116]. Tamoxifen stimulates autophagy by increasing the intracellular level of ceramide, which inhibits mTOR activation and/or stimulates expression of Atg genes.

The second generation of selective histamine H1-receptor antagonist astemizole is a potent inducer of autophagy at biologically achievable concentrations [117]. Astemizole exhibits antifungal activity and antimalarial properties making it attractive option for CF patients even though the mechanism by which it activates autophagy is still unclear [117, 118]. Safety and drug interaction profiles of astemizole are well characterized. However, due to the availability of superior next-generation histamine receptor agonists, it is not commonly used in Europe or North America.

The potential application of the TGM2 inhibitor cystamine in CF patients has recently been reviewed. Cystamine restores normal autophagy in CFTR-deficient cells and mouse models. Cystamine also restores normal trafficking of the F508del-CFTR and stabilizes the expression of the protein at the plasma membrane of airway epithelial cells [100]. In a pilot clinical trial

involving 10 F508del-CFTR homozygous CF patients, the combination of cysteamine and epigallocatechin gallate (EGCG) restored the levels of the autophagy molecules BECN1 and p62 and improved CFTR function from nasal epithelial cells *in vivo*. These effects correlate with a decrease in chloride concentrations in sweat [119]. Although the mechanism of EGCG-mediated autophagy is unclear, it seems like a viable option for targeting autophagy in CF. Depletion of p62 in CF cells disintegrates the mutant CFTR aggregates and releases sequestered molecules such as BECN1, improving overall autophagic flux in the cell. This was accompanied by improved bacterial clearance [30, 31]. This indicates that targeting p62 in CF is a promising approach to improve bacterial clearance and reduce inflammation in CF [30, 31]. Since the expression of several autophagy proteins is low in CF cells, it is necessary to restore their levels to effectively improve autophagy activity. Identifying *Mir17* and *Mir20a* as new targets to improve the expression of autophagy proteins and CFTR function offers a viable target in the CF field. Further studies should explore whether other epigenetic regulatory elements contribute to low expression of autophagy proteins in CF.

11. Concluding remarks

There is now strong evidence that immune cells, such as macrophages and neutrophils, are intrinsically impaired in CF. We therefore, recommend that CF be added to the list of innate immune disorders. In fact, autophagy, an intracellular degradation process that contributes to bacterial clearance, protein degradation, and cell survival, is defective in CF. Therefore, we propose that altered autophagy in CF contributes to chronic lung infection and inflammation. The CF field is in desperate need for an approach to correct autophagy in CF patients. The autophagy-correcting agents should ameliorate the marginally positive effects of correctors (therapies that correct the trafficking defect of F508del CFTR) and activators (compounds that activate the F508del CFTR that reaches the cell membrane) typically used in CF. Thus, the development of safe novel autophagy stimulating agents will improve the clinical outcome of CF and promote the clearance of infectious agents.

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