Dysregulated macroautophagy and abnormal mitochondria in Cystic Fibrosis

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

University of Leeds

Faculty of Medicine and Health

May 2020

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Disclaimers

The candidate confirms that the work is his own and that appropriate credit has been given where reference has been made to the work of others.

I would like to dedicate this thesis to my parents,

Alan and Jane Holbrook,

to whom I owe everything.

Without their constant love and support,

none of this would have been possible.

Acknowledgments

A PhD is not something you can possibly undertake on your own without a huge amount of help from other people. There are so many people I am grateful to who gave me advice, or just provided me with a kind word when things were not going to plan in the lab, but I'll limit myself to a select few.

First, to my three supervisors. I would like to thank Professor Daniel Peckham for all his kind words and support throughout. I greatly admired his passion for research, his unwavering positivity and encouragement made the work a lot more enjoyable. I am also extremely grateful to Dr Sinisa Savic, for his honest advice and insight into how the project should progress, his scientific critique has proven vital to improving the quality of my work. Finally, I would like to express my sincere gratitude to Professor Michael McDermott, who gave me invaluable advice throughout the process and has greatly helped me to become a better writer and researcher. His guidance, support and willingness to read everything I wrote has been instrumental to my work and I will always be thankful to him.

Dr Samuel Lara Reyna, Dr Heledd Jarosz Griffiths, Dr Chi Wong and Dr Thomas Scambler, I can't thank enough for the endless time and effort they put in to helping my project progress, this project truly would not have been possible without them. I greatly enjoyed going through this entire process with all of them, with all the conferences, nights out, and lab meetings, they made them a lot more fun. I am extremely thankful to my other friends and work colleagues, Dr Shelly Pathak, Mr Tobias Russell, Dr Payal Ganguly, Mr Georgi Georgiev and Ms Hannah Rowe. Their friendship and daily coffee breaks with copious amounts of coffee and biscuits has helped me immensely to complete this huge undertaking, I will be forever grateful that I ended up in the same office as all of them.

I would also like to express my gratitude to the research nurses at the Adult Cystic Fibrosis Unit at St. James's Hospital, Leeds, with a special thanks to both Lindsey Gillgrass and Anne Wood, their tireless efforts for the CF community will always be appreciated.

Finally, to my better half, Nadin Fathallah, or as she is now called, and I am incredibly proud to say, Dr Nadin Fathallah. I cannot thank her enough for always being there for me when things got tough, for

never failing to cheer me up and make me laugh after a long frustrating day in the lab. Her unwavering love, support and understanding whenever I came home at a horrendously late hour from work has made this whole thing possible.

Abstract

Cystic Fibrosis (CF) is an autosomal recessive genetic disorder, caused by mutations in the CF Transmembrane Conductance Regulator (CFTR) gene, which encodes an ion channel protein that conducts chloride ions, bicarbonate, and regulates the function of other channels. This CFTR protein, when defective, results in excessive pulmonary and systemic inflammation, recurring bacterial infections and progressive lung disease. Autophagy is an essential cellular process by which damaged organelles, as well as misfolded and aggregated proteins, are cleared from the cell. This mechanism is postulated to be defective in CF, as a result of the mutated ΔF508 CFTR protein; however, the current model as to how autophagy is dysregulated remains a subject of debate.

In this thesis I have investigated the state of the autophagy-lysosomal axis in CF, using human bronchial epithelial cell lines (HBECs), as well as monocytes and M1 macrophages from patients with homozygous ΔF508 CFTR mutations. I have also examined the effects of currently available CFTR modulators on modifying this axis. The state of mitochondria was also investigated to establish if dysregulated autophagy leads to an accumulation of damaged mitochondria, thereby exacerbating the NLRP3 inflammasome-driven inflammatory phenotype in CF. The impact of this dysregulation on metabolism was also investigated.

This study found autophagy to be dysregulated in cells with CF mutations. In CF HBECs, there was an increased turnover of autophagy markers in HBECs homozygous for ΔF508 CFTR. CF monocytes and M1 macrophages, however, showed an increase in a ubiquitin binding protein, p62, with no clearance of the protein via autophagy, but normal clearance of autophagosomes. CFTR modulators, Symdeko and ivacaftor, failed to show an improvement in autophagic function in all cell types. CF monocytes were found to have damaged and damage-prone mitochondria, as well as having an increased metabolic profile. These studies suggest that autophagy is dysregulated in cells that are homozygous for ΔF508 CFTR, and that the mitochondria in these cells are more prone to damage, with an increased metabolic profile.

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Glossary of Abbreviations

ABC	ATP-binding cassette
A. fumigatus	*Aspergillus fumigatus*
ASC	Apoptosis-associated spec-like protein
ASL	Airway surface liquid
Atg	Autophagy related gene
ATP	Adenosine triphosphate
BALF	Bronchial alveolar lavage fluid
B. cepacia	*Burkholderia cepacia*
BSA	Bovine serum albumin
BEGM	Bronchial Epithelial Growth Medium
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductor regulator
CK2	Casein kinase 2
Cl^-	Chloride ion
DAMP	Danger-associated molecular patterns
ER	Endoplasmic reticulum
ENaC	Epithelial sodium channel
FBS	Foetal Bovine Serum
GSH	Glutathione
HCO_3^-	Bicarbonate
HBEC	Human bronchial epithelial cell
Hsp	Heat shock protein
ICAM-1	Intercellular adhesion molecule-1
IκB-β	IkappaB-β
IL	Interleukin
LPS	Lipopolysaccharide
LTDR	Lysotracker Deep Red
LXA_4	Lipoxin-A_4
mROS	Mitochondrial ROS
MTDR	Mitotracker Deep Red
MTG	Mitotracker Green

NBD	Nuclear binding domain
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-kappaB
NO	Nitric oxide
P/S	Penicillin and streptomycin
P. aeruginosa	*Pseudomonas aeruginosa*
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PIASγ	Protein inhibitor of activated STAT
PM	Plasma membrane
PKA	Protein kinase A
PVDF	Polyvinylidene Difluoride
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
S. aureus	*Staphylococcus aureus*
sTREM-1	Soluble triggering receptor expressed on myeloid cells-1
TG2	Transglutaminase 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TMD	Transmembrane domain

Chapter 1.0 – Introduction

1.1 Cystic Fibrosis

Cystic Fibrosis (CF), is one of the most common life-limiting recessive genetic disorders in Caucasians, affecting 1 in 2500 people of European descent. A total of 70,000 people are affected worldwide, with a large variation in disease prevalence between different ethnic groups [1]. CF results from mutations of the *CFTR* gene, located on the long arm of chromosome 7, which codes for the CFTR protein, a 1480 amino acid polypeptide [2-5]. Since the localisation of the CF locus to human chromosome 7cen-q22 in 1985 [6], and the discovery of the mutated *CFTR* gene in 1989 [2, 3, 5], over 2000 mutations have been identified. At least 1500 of these mutations are associated with the CF phenotype [7], the most common mutation being the ΔF508, which occurs in 70 - 90% of CF cases in a European population [8]. CF-associated mutations are split into seven distinct categories [9], based on the various mechanisms by which they disrupt the synthesis, trafficking and function of CFTR, with associated pathology (Table 1.1). The most common mutation, ΔF508, results from the deletion of three nucleotides leading to the deletion of a phenylalanine (F) residue at position 508, and impaired folding of the CFTR protein [2]. However, due to the wide variation in disease phenotype in patients with homozygous ΔF508, the link between disease severity and the different *CFTR* mutation categories has been difficult to establish [10, 11], and both epigenetic and environmental factors have been suggested to play a role [12-14].

Class	Example Mutation	Molecular impairment	Cell line
I	W1282X	CFTR not synthesised	IB3-1
II	ΔF508	Faulty protein processing	CuFi-1, CFNPE14o-
III	G551D, R117H	Faulty channel function	CuFi-4, CFBE45o-
IV	R347H, R117H	Altered conductance	CFBE45o-
V	621+1G>T, R117H	Faulty splicing	CFBE45o-
VI	S1455X, ΔF508	Reduced stability	CuFi-1, CFNPE14o-
VII	Dele2,3(21kB), 1717-1G	No mRNA, no protein	Complete knockout

Table 1.1 – CFTR classification based on molecular impairment. The mutations shown are put into classes based on what molecular impairment they cause in the CFTR protein. Many mutations have more than one class of action. Also showing what cell line is available for each class of mutation, with some mutations resulting in two or more molecular impairments [9, 15].

As the CFTR protein is primarily found on the apical membrane of epithelial cells of the respiratory tract, gastrointestinal tract, reproductive organs and the sweat ducts, its mutated form results in a multisystem disease affecting many organs, but lung disease remains the most common cause of morbidity and mortality [16]. The condition is characterised by progressive lung involvement with bronchiectasis, airflow obstruction, recurrent bacterial infections, and a heightened inflammatory response, as demonstrated by increased levels of cytokines in the sputum, bronchial alveolar lavage fluid (BALF) and lungs of patients with CF [17]. There is a wide range of other pathological phenotypes, as a result of the CFTR being expressed on such a broad range of cells; disease phenotypes include pancreatic insufficiency, malabsorption, malnutrition, CF related diabetes, bowel obstruction, and male infertility [15]. Defective CFTR largely affects the function of epithelial tissues, where the protein is highly expressed, predominantly on the apical membrane of epithelial and secretory cells, such as human bronchial epithelial cells (HBECs) and luminal cells of the intestine and pancreas [2]. CFTR protein is also expressed on a range of other cell types, such as monocytes, lymphocytes [18] and neurons [19].

CFTR is a membrane-spanning protein, expressed on the apical side of epithelial cells, and is the only ion channel to be a member of the adenosine triphosphate (ATP)-binding cassette (ABC) family of proteins, containing a regulatory (R) domain that is actively phosphorylated by protein kinase A (PKA) [20-22] (Figure 1.1). Once PKA has phosphorylated the R domain, this allows the nuclear binding domains (NBDs) to interact with ATP, leading to a conformational change in the transmembrane domains (TMDs), which results in the channel opening with efflux of Cl^-, bicarbonate (HCO_3^-) ions and glutathione (GSH). The CFTR is also able to sense Cl^- concentration, regulating its own activity, as increased Cl^- concentration in the luminal space leads to a reduction in the ATPase activity of the CFTR [23]. The ΔF508 mutation occurs within a key region of NBD1, destabilising the domain, distorting the inter-domain assembly and, therefore, the communication of the transmembrane domains necessary for channel opening [24, 25]. The mutated CFTR protein is marked for degradation

in the proteasome and following proteasome overload, a limited amount of CFTR protein, with defective gating and stability, reaches the plasma membrane [26].

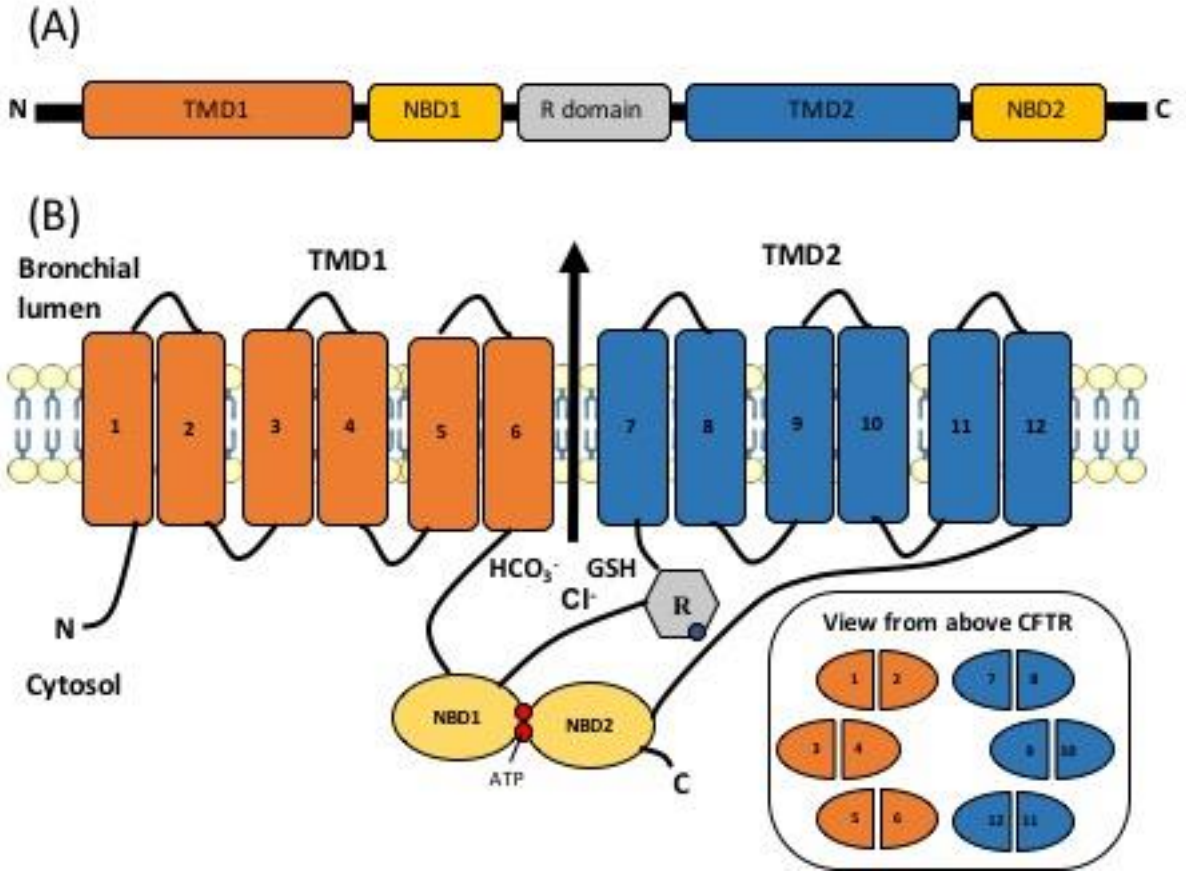


Figure 1.1 – (A) Linear structure of the cystic fibrosis conductance regulator (CFTR). From N to C terminal, transmembrane domain (TMD)1, nuclear binding domain (NBD)1, regulatory (R) domain, TMD2 and NBD2. **(B) Structure of the CFTR ion channel.** The ion channel consists of 2 membrane spanning domains, TMD1 and TMD2, each domain is split in to 3 regions of 2 subunits, each domain being connected to a NBD as well as the R domain. When the R domain is phosphorylated by protein kinase A and ATP binds to the NBDs, the channel opens, allowing the transport of mainly Cl^- but also HCO_3^- and GSH out of the cell. Adapted from Ratjen *et al* 2015 [15]. Figure produced using Smart Servier Medical Art.

Defective or absent CFTR results in diminished, Cl^- ion efflux and reduced inhibition of the epithelial sodium channel (ENaC), resulting in increased Na^+ influx [27, 28]. This increase in Na^+ flux across the apical to basolateral membranes results in increased water absorption, reduced airway surface liquid (ASL), increased sputum viscosity and impaired mucociliary clearance (Figure 1.2) [29]. The importance of the ENaC channel in CF pathology is demonstrated by studies in a CF mouse model where overexpression of the β-ENaC chain results in a CF-like lung phenotype [30]. The lack of HCO_3^- and GSH secretion also plays a role in the pathology, as it leads to an increase in the acidity of the ASL.

The reduction in pH can reduce cilia beat frequency [31], and impair bacterial killing, via phagocytic cells [32], and defective defensins, a highly pH-sensitive protein [33].

In healthy individuals, the layer of ASL and mucus serves as an essential barrier against pathogens and other environmental stimuli, such as dust, pollen and pollution. The mucus traps pathogens and foreign particles which are then removed by the mucociliary escalator, which propels mucus up out of the lungs and into the pharynx where it can be swallowed. However, in the CF lung, the combination of ASL dehydration and abnormal thick mucus results in defective mucociliary clearance and a predisposition to acute and chronic bacterial infections. Infection also triggers an abnormal immune response which results in hyperinflammation and tissue damage (Figure 1.2). These recurrent opportunistic infections are caused by a range of microbes, the most common being *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Haemophilus influenzae*,

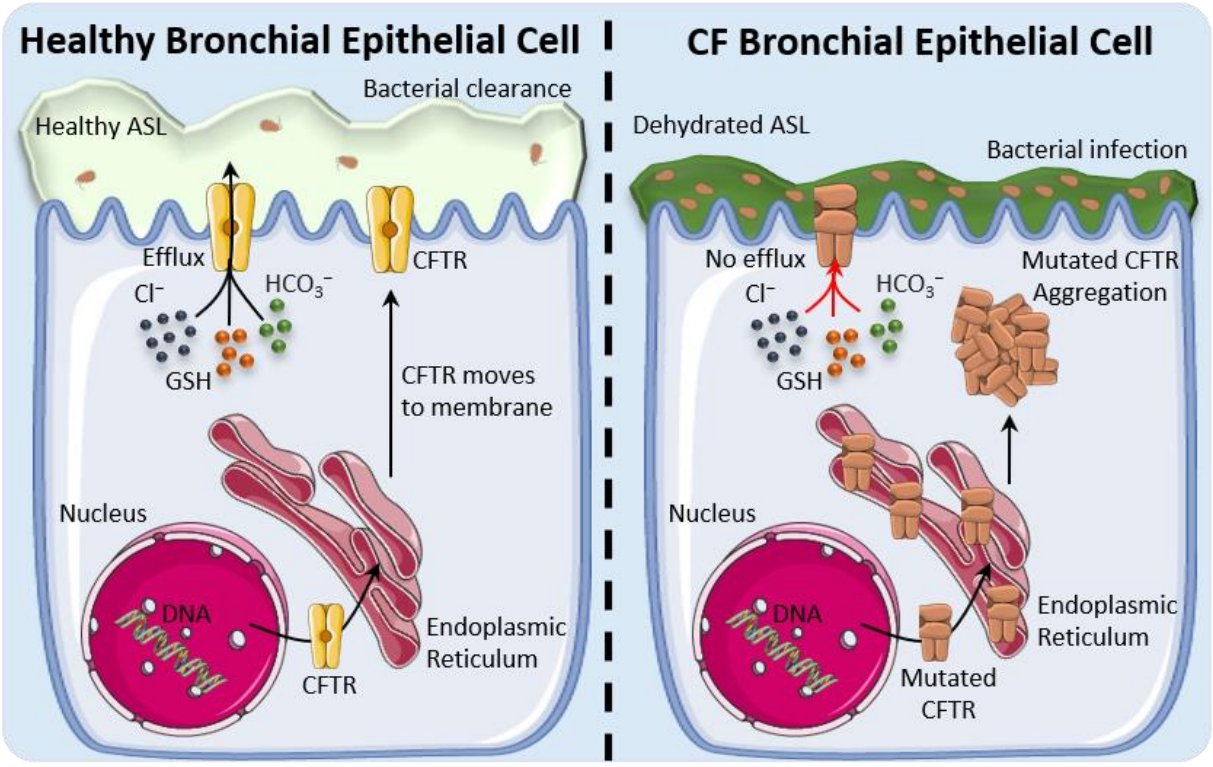


Figure 1.2 – Pathophysiology of Cystic Fibrosis (CF) human bronchial epithelial cells (HBECs). In healthy cells, after the CFTR is transcribed and translated, it undergoes post-translational modifications in the endoplasmic reticulum (ER), after which it is transported to the cellular membrane to facilitate the efflux of Cl^-, GSH and HCO_3^- into the extracellular space. This maintains the airway surface liquid (ASL) height and reduces its viscosity, aiding in bacterial clearance via the mucociliary elevator and allowing appropriate immune cell migration. However, in CF the class II mutated CFTR (the focus of this thesis) is either retained in the ER, forms aggregates in the cytosol, or moves to the cell membrane where it is unable to facilitate efflux. This leads to a dehydrated ASL and impaired immune response, providing an optimum environment for infection by various pathogens. Figure produced using Smart Servier Medical Art.

Burkholderia cepacia (*B. cepacia*) complex and *Stenotrophomonas maltophilia (S. maltophilia*) [34]. Fungi such as *Aspergillus fumigatus* (*A. fumigatus*) and viruses such as rhinovirus are also associated with pulmonary exacerbations and clinical deterioration [35, 36].

In addition to ENaC, the CFTR protein also acts as a regulator of other ion channels, including Cl^- exchangers and other Cl^- channels, such as anoctamin 1 (Ca^{2+} dependent Cl^- channel) [37]. Cell surface retention of the CFTR is also regulated by cytosolic Ca^{2+}, as an increase in Ca^{2+} results in a decrease in CFTR conductance as well as membrane expression [38]. Other secondary functions include, aiding phagocytosis by immune cells [39, 40], and acting as a receptor for pathogens to enter the cell, such as *Salmonella typhi* (*S. typhi*) in the intestine [41], *Chlamydia trachomatis (C. trachomatis)* [42], and *P. aeruginosa* [43].

1.1.1 CFTR protein folding and trafficking

The *CFTR*, once translated into a peptide, follows the protein trafficking pathway in a similar fashion to other plasma membrane (PM) proteins, encountering a number of quality control checkpoints, which ensure the protein is in its native conformation [44]. The first checkpoint is on the ER membrane, where the newly translated polypeptide CFTR is inserted through the ER membrane translocon (Sec61 complex) by the use of the signal recognition particle receptor [45]. The CFTR's core is then glycosylated at Asn894 and Asn900 amino acid residues [46], the lectin/chaperone calnexin assesses whether the glycosylation was successful, and calreticulin is then used to examine its conformation, to ensure its proper folding. The heat shock proteins (Hsp), Hsp40, Hsp70 and Hsp90, play vital roles in the quality control process for protein folding, and have recently been shown to act as a 'chaperone trap' and associate with the ΔF508 CFTR but not wild type (WT) CFTR [47]. This is due to varying binding affinity, as the exposed hydrophobic residues on the ΔF508 CFTR surface allow for stronger binding [48].

After post-translational modifications at the ER, the CFTR is transported to the Golgi complex, a point where ΔF508 CFTR is not able to proceed, due to its ER retention motifs, consisting of four

arginine-framed tripeptides, which keep the ΔF508 CFTR at the ER membrane. The WT CFTR does not have this retention motif exposed; however, the protein also requires the presence of a di-acidic code (DAD) motif, located on NBD1, which acts as a positive signal for the CFTR to be moved to the Golgi complex [49]. Once at the Golgi, the protein moves through the complex by a number of Golgi glycosyltransferases, developing the CFTR into its fully mature form [50]. The largest branch of the Ras superfamily, Rab GTPase [51], plays a vital role in the final step of delivering the mature CFTR to the PM. The mature CFTR then exits the Golgi complex in vesicles, which are transported along actin cytoskeleton fibres to the PM, a process facilitated by myosin Vb and controlled by Rab11 [52]; however, in ΔF508/ΔF508, the faulty CFTR is largely retained in the ER. Any mutated CFTR that does make it to the PM is marked for endocytosis via ubiquitination, as the incorrectly folded CFTR is bound by chaperones, for prolonged periods of time, prior to being endocytosed [44]. Peptide fragments, which are proteolytically cleaved from the ΔF508 CFTR, whilst it is being transported to the cell membrane, trigger the overactivation of the multifunctional enzyme, casein kinase (CK)2. CK2, in its overactive state, then phosphorylates the CFTR at position Threonine 1471, destabilising it further and causing it to fragment [53].

1.1.2 CFTR therapeutics

Several medical therapies are available to treat lung disease in patients with CF, and these are largely focused on preventing and controlling symptoms rather than treating the underlying pathophysiology. These include hypertonic saline to improve mucociliary clearance, anti-inflammatory agents, such as low dose macrolide antibiotics and high dose ibuprofen, inhaled antibiotics to reduce bacterial exacerbations, and dornase alfa (pulmozyme), which reduce mucus viscosity [15].

Due to a number of recent breakthroughs in the understanding of the transport and folding of CFTR, new small molecule therapies have come to market that target different cellular mechanisms, to either improve the function of the channel (potentiators) or to facilitate the transport of the CFTR

to the membrane (correctors). The CFTR potentiator, ivacaftor (VX-770), was the first highly effective CFTR modulator to become available for the treatment of CF. The drug acts by binding to NBD1 and NBD2 and forcing the channel to open; because it is a potentiator, it can only work on gating and residual functioning mutations, where the CFTR protein is expressed on the cell membrane but is functionally abnormal [54]. Due to its proven safety and efficacy, ivacaftor has been approved for use in most countries for patients with the class III mutations, including G551D and residual function variants such as Arg117His-CFTR [55, 56]. The combination of ivacaftor with the corrector lumacaftor (VX-809) is available in some countries as the combination pharmaceutical drug, Orkambi, for patients with class II mutations, with demonstrated improvements in lung function but with a higher rate of drug intolerance [57-60]. Tezacaftor (VX-661) is a newer CFTR corrector which when combined with ivacaftor (brand name Symdeko) has similar efficacy to Orkambi but with fewer side effects and different anti-inflammatory properties [61-63]. More recently, two new modulators have been developed, VX-659 and elexacaftor (VX-445); both have separately been combined with tezacaftor/ivacaftor to form a triple therapy, which has recently been tested in Phase III clinical trials [64, 65]. The elexacaftor combination has recently been approved for use in the USA under the brand name Trikafta, and is considered a significant step forward in the treatment of CF, as it has been shown to be highly efficacious and has the potential to effectively treat up to 90% of patients, including patients who are either heterozygous or homozygous for the ΔF508 mutation [64, 65]. The initial results from Phase III clinical trials have shown that triple therapy is significantly more efficacious than double therapy at increasing lung function and weight, reducing pulmonary exacerbations and downregulating sweat Cl^- [64]. Some rare CFTR mutations, such as R560S, show no improvement in function with any of the current CFTR modulators [66].

Other triple combinations, such as the use of pharmacological chaperones, lumacaftor, MCG1516A and RDR1 in combination have also been investigated, and have demonstrated better CFTR recovery than lumacaftor on its own [67].

1.2 Inflammation in CF

CFTR is expressed in various immune cells [68-78], and when defective, as in CF, results in an abnormal immune response. The innate immune system is hyperactive in CF, leading to an excessive inflammatory state persisting from a young age; however, the debate as to whether infection is required for airway inflammation in CF is still ongoing. The dysregulation of the self-degradative process called autophagy [79], an irregular unfolded protein response (UPR) [80] and a hyperactive NLRP3 inflammasome [28] have both been shown to lead to an inflammatory state in CF, due to the lack of function and associated accumulation of mutated CFTR. Neutrophil-driven inflammation has also been shown in CF lungs, in the absence of infection [81], and a recent animal model study has indicated that the abnormalities present in the mucus layer, similar to those found in CF, can be inflammatory in nature in the absence of infection [82]. Despite this ongoing debate, it is well established that the CF lung is hyperresponsive to inflammatory stimulus, such as infection, pollution, dust etc., thereby leading to a chronic inflammatory state.

The presence of high levels of inflammatory cytokines and chemokines in the lungs, sputum, BALF and serum of patients with CF is well established; the cytokines and chemokines include interleukins (IL)-1β, IL-6, IL-8, IL-17, IL-33, tumour necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF and high mobility group box 1 (HMGB-1) protein [83-85]. Furthermore, the inflammatory marker, soluble triggering receptor expressed on myeloid cells (sTREM)-1, which is expressed on neutrophils, monocytes, macrophages and endothelial cells, and acts by enhancing Toll-like receptor (TLR) signalling pathways [86], has been reported to be raised in CF [87, 88]. The expression of both matrix metalloproteinase (MMP)-8 and MMP-9, which cleave TREM-1 to form sTREM-1, were also found to positively correlate with increased sTREM-1 expression [87]. In addition to this, levels of anti-inflammatory/pro-resolution molecules, such as IL-10, lipoxin-A_4 (LXA_4) and nitric oxide (NO) have all been shown to be reduced in CF BALF [83, 89, 90]. This increase in inflammatory cytokines results in chronic inflammation; for example, the increase in GM-CSF both inhibits apoptosis of neutrophils as well as their clearance from the lungs [91], whereas the deficiency

of anti-inflammatory/pro-resolution molecules allows inflammation to persist, as for example, IL-10 would normally inhibit production of inflammatory transcription factors, and thus, the associated cytokines, and induce neutrophil apoptosis [83].

Once an infection is present in the CF lung, innate immune cells, such as neutrophils and macrophages, migrate to the site of infection, as part of an excessive inflammatory response. Neutrophils are responsible for the earliest innate immune response to infection and play a significant role in the inflammatory phenotype of the CF lung [92]. In addition high levels of the chemokine, IL-8, attracts an excessive neutrophil infiltration of the lungs [93]. As neutrophils come into contact with the pathogen, they release toxic oxidants, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), to kill the microbes, with subsequent removal of dying cells by efferocytosis, the process by which apoptotic cells are removed by phagocytic cells. They also release DNA fibres with antimicrobial proteins attached, forming neutrophil extracellular traps (NETs). These act in a bactericidal way to remove the pathogen; however, in CF, excessive NET formation may lead to a non-resolving cycle of inflammation [94]. Chronic neutrophilic inflammation in CF is also characterised by the production of factors that greatly reduce ASL height, thereby exacerbating airway dehydration [95]. The imbalance between some of these factors, such as elastase, neutrophilic protease enzymes and their regulatory counterbalancing factors, anti-proteases, leads to severe tissue damage, as the removal of apoptotic cells becomes impaired. This damage occurs as apoptotic recognition receptors on phagocytes, such as the phosphotidylserine receptor, are cleaved by elastase [96]. An animal model study, in 2014 [97], examined the ability of *CFTR*$^{-/-}$ mice to clear bacterial infections, and found that despite the neutrophilic influx and release of bactericidal elements, the infection still persisted. The complement system, another important component of innate immunity related to neutrophils, has also been shown to be dysregulated in CF [98]. The C3b receptor, CR1, which should be upregulated after neutrophils are activated at the site of infection, is proteolytically cleaved *in situ*, interfering with effective phagocytosis, and, therefore, stunting the ability of the innate immune system to remove pathogens [98]. Neutrophils in CF also demonstrate high levels of adherence to airway epithelia, which

is associated with raised levels of IL-6 and IL-8, contributing to the chronic inflammation found in the CF lung [99]. Neutrophils have also been shown to have delayed apoptosis, which contributes to overproduction of NETs and the resulting tissue damage [100].

Monocytes are essential to a functioning immune system; they originate in the bone marrow, are capable of phagocytosing invading pathogens, secreting cytokines, and also, have the potential to differentiate into either one of the main antigen-presenting cells, macrophages and dendritic cells. In CF, however, monocytes have been shown to be dysregulated, with abnormal expression of genes and receptors [101], such as TLR4 [102], as well as a heightened inflammatory response to lipopolysaccharide (LPS) with excessive secretion of IL-6 and TNF [103, 104], and LPS and ATP challenge with caspase-1 activation, and IL-1β and IL-18 production [28]. Interestingly the CFTR modulator, Symdeko has been shown to significantly reduce the levels of monocyte IL-1β, IL-18 and caspase-1 activation in patients over a period of 3 months, whereas Orkambi fails to lower IL-1β [63]. There have been some contradictory results published regarding the expression of surface markers and signalling pathway proteins, with one study reporting a downregulation in the expression of CD14 and HLA-DR, along with impaired phagocytosis [105], and increased expression of IL-4Rα, IL-13Rα1, M-CSF, TLR4, TIMP-1 and Cox-2 [101], whereas another study has reported no difference in expression of the cell surface marker and LPS receptor, CD14, as well as no difference in chemokine receptor expression, except for CCL2, which is upregulated [106]. MicroRNAs, encoded by the X chromosome, have been shown to be functionally increased in CF monocytes which are homozygous for ΔF508 compared to HC monocytes. miR-224-5p was the most upregulated, with its downstream target SMAD family member 4 (SMAD4), which is required for endotoxin tolerance, being downregulated, indicating a potential role in inflammation in CF [107]. The programmed death-ligand 1 (PD-L1) plays a key role in the suppression of the adaptive immune arm of the immune system and has been shown to be upregulated in monocytes in CF, leading to an impaired T cell response, with low expression of HLA-DR upon infection with *P. aeruginosa*; the T cell response is recovered upon PD-L1 inhibition [108].

Monocytes from CF patients, with at least one G551D mutation, show increased expression of the cell surface markers, receptor activator of nuclear factor κB (RANK), which is a member of the TNF receptor family, and macrophage-colony stimulating factor receptor (M-CSFR); overexpression of these markers was decreased after 9 and 12 months of ivacaftor treatment [109]. Another study using patients' monocytes with a G551D mutation with ivacaftor treatment showed the impact ivacaftor has on the proteome over a period of 7 days, finding a reduction in inflammatory proteins induced by interferon (IFN)γ [110].

Macrophages play another key role in the innate immune response, clearing pathogens through phagocytosis, and removing NETs and neutrophils themselves by a process called efferocytosis. Macrophage targeting of pathogens and necrotic cells occurs through the engulfment of the targeted pathogen or necrotic cell by the macrophage, encapsulated within a vacuole, the vacuole then moves to and fuses with a lysosome for degradation. However, lysosomes in macrophages in CF have been shown to be defective, as they are able to fuse with vacuoles but not able to acidify its contents, resulting in exacerbation of lung inflammation [111, 112]. However, some studies have failed to confirm the link between faulty CFTR and acidification [113, 114]. Another study has also shown that macrophage phagocytic activity is not defective, despite the CFTR mutation, although the number of surviving intracellular bacteria was greater in CF than in the healthy control (HC) [73]. Macrophage TLR4 trafficking and degradation is defective in CF, which leads to overactivation of nuclear factor-kappaB (NF-κB), MAPK and IRF-3 pathways and retention of TLR4, when stimulated with LPS, and this results in an exaggerated inflammatory response to various stimuli [115]. It has also recently been shown that the populations of M1 and M2 macrophages in the CF lung are abnormal, as there is an increased number of macrophages of the more inflammatory M1 phenotype, with a reduced number of pro-resolution M2 macrophages present compared to HCs [116]. The UPR response in alveolar macrophages in CF has also been shown to be dysregulated via the upregulation of the IRE1α arm of the UPR, leading to excessive splicing of XBP-1 and heightened TNF and IL-6 production [117]; this has also been replicated in monocytes and M1 macrophages [103].

CF monocyte-derived macrophages have been shown to have differential reactions to the addition of ivacaftor, or ivacaftor with lumacaftor, with increased CFTR expression and decreased apoptosis. However, only ivacaftor, on its own, was able recover CF macrophage polarisation, decrease inflammatory cytokine production, improve phagocytosis and reduce the bacterial burden of *P. aeruginosa* [118]; this indicates, along with the clinical data involving lumacaftor, that lumacaftor has some adverse off target effects.

Despite the large influx of innate immune cells to the site of infection in the CF lung, this response fails to eradicate the invading microbes, which leads to excessive inflammation, demonstrating that CF is essentially an innate immune disorder, due to the defective innate immune cells.

Another part of the innate immune response, involving bronchial epithelial cells, plays a role in the innate immune response to infection; however, in CF, the response to stimulation is excessive, such as the disproportionate activation of NF-κB and the inappropriate processing of IkappaB-β (IκB-β), resulting in excessive secretion of inflammatory cytokines [119, 120]. Excessive NF-κB activation has been partly linked to Ca^{2+} in airway epithelial cells in CF [121, 122], and to a hyperinflammatory response to infection [123]. Expression of the IL-8 chemokine, and also, the leukocyte adhesion glycoprotein and intercellular adhesion molecule-1 (ICAM-1), is raised, promoting the migration and occupation of the lungs by neutrophils [124]. This mechanism of a disproportionate response is common among a number of autoinflammatory diseases of the lung, such as chronic obstructive pulmonary disorder (COPD), pan-bronchiolitis (DPB), non-CF bronchiectasis, CF and rheumatoid arthritis (RA) [125] (Figure 1.3). The presence of oxidative stress in CF HBECs compared to HC, such as ROS production and apoptosis, also plays a contributing role in the secretion of IL-6 and IL-8 [126].

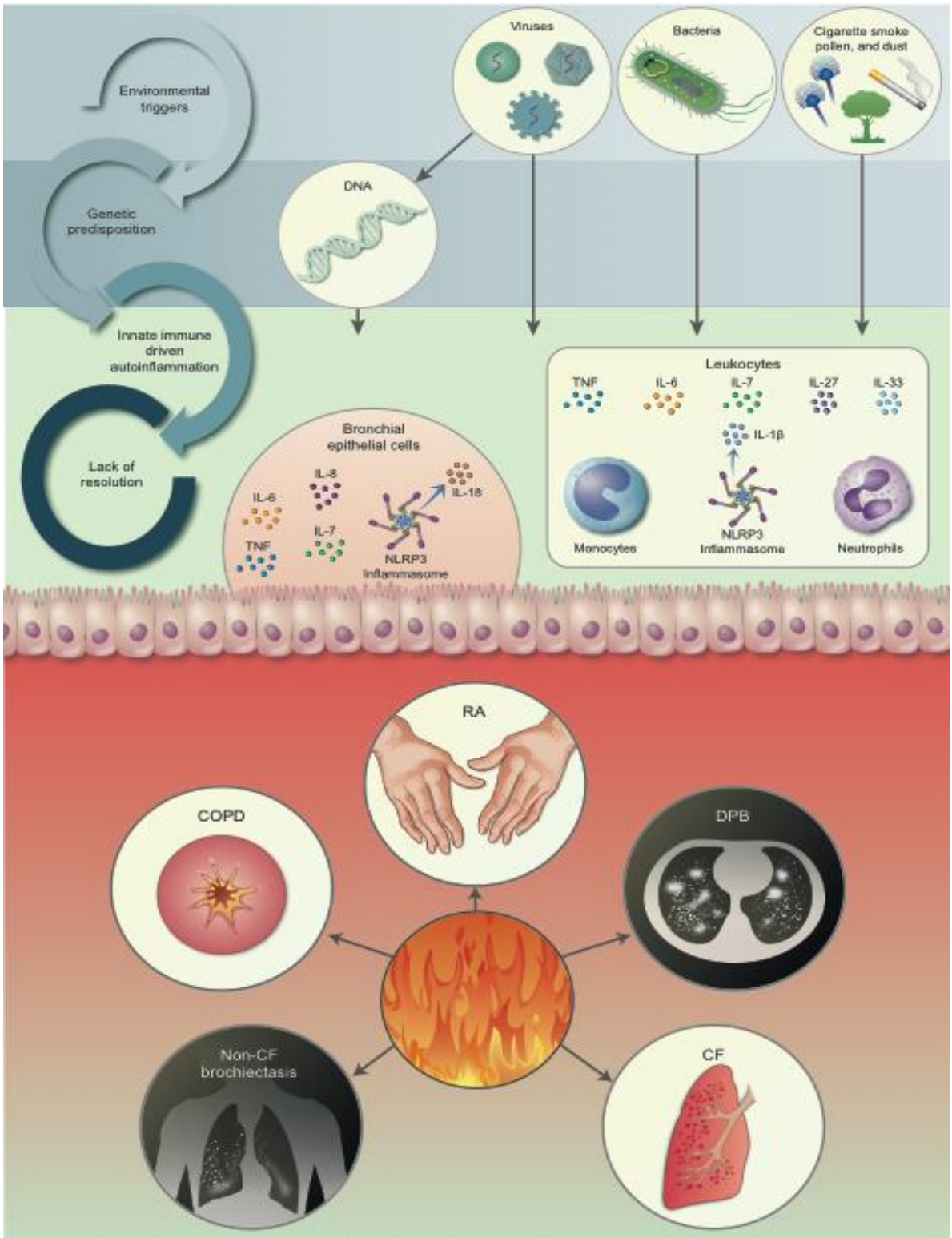


Figure 1.3 – Non-resolving cycle of autoinflammation in the lung and its related diseases. Chronic inflammation of the lungs results from the accumulation of a number of different factors, ranging from environmental insults (cigarette smoke, viral, bacterial, air pollution etc) to genetic predisposition; these cycles feed into each other resulting in innate immune driven autoinflammation and ultimately results in chronic inflammation of the lungs without resolution. A number of innate immune cells, such as neutrophils, monocytes and epithelial cells of the bronchi play pivotal roles in the excessive response to stimuli and combine to secrete a range of inflammatory cytokines. Cystic fibrosis (CF), Rheumatoid Arthritis (RA), Chronic Obstructive Pulmonary Disorder (COPD), Diffuse Pan-Bronchiolitis (DPB). Taken from Scambler, T., Holbrook, J., Savic, S., Peckham, D., McDermott, M., Autoinflammatory Disease in the Lung. Journal of Immunology [125].

In recent years, it has been shown that the NLRP3 inflammasome plays a central role in the pathogenesis of CF [127], and also in a host of autoinflammatory diseases, which can be treated with IL-1 blockade, by the IL-1 receptor antagonist (IL-1Ra), or with anti-IL-1β antibodies [128-132].

The NLRP3 inflammasome is a multimeric protein complex, which assembles in response to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), to proteolytically cleave pro-IL-1β and pro-IL-18 to their mature inflammatory cytokines, IL-1β and IL-18 [133]. A number of different inflammasomes are present in the human body [133]; however, the NLRP3 inflammasome [134] is one of the main inflammasomes involved in CF, along with NLRC4 [135]. In its active form, IL-1β induces fever, sensitises neutrophils to chemoattractants and increases the expression of adhesion molecules, such as CD18. IL-1β also promotes differentiation of the naive T cell population towards a Th17 phenotype, in the presence of IL-6 and TGF-β [136]. By contrast IL-18 has been shown to negatively regulate the Th17 cell population and promote key Treg effector molecule generation, playing an important role in the regulation of intestinal inflammation [137]. Recently, it was demonstrated that platelet count correlates with IL-1β concentration in plasma, that platelets increase NLRP3 inflammasome activation in human macrophages and neutrophils, and are essential for IL-1β production in monocytes. Furthermore, increased platelet expression has been reported in several IL-1β driven autoinflammatory diseases [138]; it would be highly interesting to establish if this is the case in CF.

Some recent studies have furthered our understanding of the role that the NLRP3 inflammasome plays in CF. One study has demonstrated that the CFTR regulated intracellular Ca^{2+} homeostasis in HBECs, with increased intracellular Ca^{2+} levels in CF HBECs, upon stimulation with *P. aeruginosa*, leads to increased mitochondrial Ca^{2+} levels and exacerbation of the NLRP3 inflammasome response to infection [139]. A more recent paper has demonstrated the role that ENaC plays in activation of NLRP3, with increased intracellular Na^{+} leading to an efflux of K^{+} from the cells, thereby acting as a trigger for NLRP3 activation and IL-1β and IL-18 production [28]. These findings suggest that ENaC modulators could be used as novel anti-inflammatory therapies in CF [140, 141].

Although CF showed features of an autoinflammatory disorder [142], both T cells and B cells also play a role in the pathogenesis of the disease, as both cell types express the CFTR and are found in large concentrations in the lungs. B cells have been shown to play a large part in the generation of peri-bronchial lymphoid follicles in the CF lung with IgA secretion, as well as in dysregulated IgE synthesis in the presence of *A. fumigatus* infection [143]. The Th2 and Th17 cell populations have recently been shown to be the prominent T cell subsets in CF, resulting in raised IL-5, IL-13, IL-17A and IL-17F in the sputum of patients with CF, which promotes both granulopoiesis and recruitment of neutrophils to the site of inflammation [144, 145]; by contrast, CD4+ T cells secrete less IL-10 than healthy T cells [146].

1.3 Other autoinflammatory diseases

Several autoinflammatory diseases bear many similarities to CF, with excessive activation of the NLRP3 inflammasome, dysregulated autophagy and UPR, as well as damaged/abnormal mitochondria. Autoinflammatory diseases, such as familial cold autoinflammatory syndrome (FCAS) [147], Muckle-Wells syndrome (MWS) [148], mevalonate kinase deficiency (MVK, also known as hyperimmunoglobulinemia D with periodic fever syndrome (HIDS)) [149], neonatal-onset multisystem inflammatory disease (NOMID) [132], Schnitzler's syndrome (SS) [150], familial Mediterranean fever (FMF) [129] and TNF receptor-associated periodic syndrome (TRAPS) [151], all add to the argument that CF is basically an autoinflammatory disease at the innate immune end of the immunological disease continuum [142, 152].

1.3.1 Familial-Mediterranean fever (FMF)

The autoinflammatory disease, familial-Mediterranean fever (FMF) is the result of a mutation of the Mediterranean fever (*MEFV*) gene, which codes for the cytoskeleton-associated protein pyrin [153] [154]. The association of mutations in pyrin with FMF was the first in a long line of susceptibility genes being linked to autoinflammatory disorders [154, 155]. Pyrin acts as a pathogen recognition receptor (PRR) in immune cells, detecting the inactivation of Rho GTPases by several bacterial toxins

via glycosylation [156]. Once Rho GTPases become inactive, due to bacterial toxins, pyrin Is activated via an unknown mechanism. This ultimately results in formation and activation of the NLRP3 inflammasome, due to the oligomerisation of pyrin with apoptosis-associated spec-like protein (ASC) and caspase-1 [157]. Recently, mitochondrial dysfunction has been identified in FMF by the detection of a significant decrease in the amount of mitochondrial DNA, an increase in mitochondrial protein damage and a mitochondrial energy deficit [158, 159]. Pyrin, which is also known as tripartite motif-containing 20 (TRIM20), has been shown to mediate selective autophagy of the inflammasome components, NLRP3, NLRP1 as well as pro-caspase-1, via recruitment of autophagic proteins Ulk1, Beclin1, ATG16L1 and LC3. The mutations found in pyrin in FMF lead to impaired selective autophagy of the NLRP3 inflammasome [160]. The basal rate of autophagy is also reduced in FMF [161].

1.3.2 TNF-receptor associated periodic syndrome (TRAPS)

TNF-receptor associated periodic syndrome (TRAPS) is caused by mutations of the *TNFR1* gene [162], which result in retention of the mutated TNF receptor in the ER, leading to excessive cellular stress, dysregulated autophagy and NLRP3 overactivation. In 2013 it was shown by Bachetti *et al* that dysregulated autophagy is a key contributor to excessive inflammation in patients with TRAPS [163]; another study has demonstrated that overproduction of mitochondrial ROS (mROS) is involved in the inflammatory state in TRAPS, as it increases inflammatory cytokine production [164]. Also, the mitochondria appear abnormal in TRAPS with increased ATP production, basal oxygen consumption rate (OCR) and an altered mitochondrial membrane potential, exacerbating the inflammatory phenotype [164].

1.3.3 Mevalonate kinase deficiency (MKD)

Mevalonate kinase deficiency (MKD) is the result of mutations in the mevalonate kinase (*MVK*) gene, a gene which is essential for cholesterol synthesis [165-167]. This mutation leads to a reduction in the levels and activity of MVK. Under normal conditions this enzyme converts mevalonic acid into mevalonate-5-phosphate, as part of isoprenoid and sterol synthesis, in MKD, however, this results in

the cholesterol biosynthetic pathway being blocked. The exact mechanism of pathogenic action is not clear in MKD; however, post-translational modifications of Rho GTPases, such as prenylation, have become interesting as potential therapeutic targets [168]. Just like in FMF, the alteration of Rho GTPases has been linked to activation of the NLRP3 inflammasome through activation of pyrin. Although the exact mechanism of pathogenic action for MKD is not known, there have been some studies suggesting a link to the UPR, as the mutated MVK protein accumulates in the cell, triggering the UPR and increasing NLRP3 activity. This is supported by the fact that NLRP3 activity is not increased in an MKD mouse model where *MVK* is knocked down, preventing MVK accumulation [169]. Furthermore, defective autophagy and abnormal mitochondria have been observed in MKD, both of which exacerbate NLRP3-driven inflammation [170]. Impaired autophagy has also been suggested to contribute to the accumulation of damaged mitochondria and oxidised mtDNA in PBMCs taken from MKD patients [171], although the same group has also shown that prenylation of RhoA failed to have any effect on autophagy [172].

1.3.4 Cryopyrin associated periodic syndromes (CAPS)

Mutations in the *NLRP3* gene have been linked to a number of autoinflammatory diseases, such as MWS [173, 174], FCAS [175], and chronic infantile neurological, cutaneous, articular syndrome (known as CINCA or neonatal-onset multisystem inflammatory disease (NOMID)) [176, 177]. The spectrum of these disorders is collectively known as cryopyrin associated periodic syndrome (CAPS). These mutations occur largely in both the nucleotide binding domain (NBD) and oligomerisation domain of NLRP3, thus altering the oligomerisation of the NLRP3 inflammasome, via changing protein-protein interactions leading to hyper-inflammation [175, 178, 179]. Monocytes in CAPS disorders have been shown to have damaged and fragmented mitochondria and raised levels of ROS, which contributes to further activation of the NLRP3 inflammasome [180].

1.3.5 Treatments for autoinflammatory diseases

The high success rate of IL-1 receptor antagonists (IL-1Ra), such as anakinra, in treating these disorders is exemplary in demonstrating the central role of IL-1β in the pathogenesis of these diseases [131, 132, 142, 148]. Somatic mosaicism has been reported in several cases of FMF [181], and TRAPS [182], as well as in a Japanese patient with CINCA/NOMID [183]. The use of IL-1Ra has been shown to be efficacious in several other autoinflammatory diseases without mutations in the *NLRP3* gene such as FMF [129], TRAPS [151] and MKD [128], although the response to these therapies in these particular diseases has been varied. Preclinical studies of IL-1Ra treatments, e.g. anakinra, in CF have some promising results, with mouse models and primary human bronchial epithelial cells showing reductions in inflammation and mucus secretion, as well as upregulated autophagy, the proteasomal pathway and clearance of pathogens [135, 184]. More recently, specific inhibition of the NLRP3 inflammasome with MCC950 has shown an improvement in clearance of *P. aeruginosa* in a CF mouse model and a reduction in IL-1β production [185].

These reports demonstrate the similarities between the inflammation found in CF and that of autoinflammatory diseases, as they are both largely NLRP3 inflammasome driven, have dysregulated autophagy as well as mitochondrial abnormalities.

1.3 Autophagy

The self-degradative cellular process, termed autophagy, is a vital pathway by which damaged organelles, misfolded or aggregated proteins and pathogens are encapsulated within a membrane and broken down within the cell [186]. Autophagy also plays an essential role in energy source recycling at times of cellular development and stress [186]. There are three main types of autophagy, chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. CMA refers to the process by which specifically targeted proteins are bound by chaperone proteins, such as Hsp70. The chaperones move the protein to the lysosome membrane to bind to the lysosomal associated membrane protein 2A (LAMP-2A), where it is translocated across the membrane, and finally degraded

[187]. This contrasts with micro-autophagy, where the lysosome directly takes up cytosolic components to be degraded, which occurs through invagination of the lysosomal membrane. Macroautophagy (Figure 1.4), henceforth referred to as autophagy, is the main mechanism by which cellular components are degraded and recycled; this process involves the encapsulation of cellular components that are to be degraded within a double membrane, called an autophagosome. The autophagosome then transports its contents to a lysosome, where it fuses to form an autolysosome, thereby degrading its contents [186]. So far 32 autophagy-related genes (Atg) have been identified in yeast, with a similar number shown to be present in a number of different organisms, such as mammals, plants, worms and flies, showing the importance of this highly conserved biological mechanism [188].

Autophagy has four main steps: initiation, phagophore formation, membrane elongation and nucleation and, finally, maturation. The initiation of autophagy is triggered by a strong stimulus, such as amino acid starvation or a shift towards ADP in the ATP:ADP balance within a cell, as detected by adenosine monophosphate kinase (AMPK); this results in inhibition of the mammalian (or mechanistic) target of rapamycin (mTOR)1 complex (mTORC1), a key regulator of autophagy, which is sensitive to hypoxia, in addition to growth factor, insulin and ATP signalling.

1.3.1 Initiation and phagophore formation

Under normal conditions mTORC1 (Figure 1.4), consisting of mTOR, regulatory associated protein of mTOR (RAPTOR), Rheb and Rag, phosphorylates both Unc-51-like kinase 1 (Ulk1) and Atg13, keeping them in inactive states. However, under starvation conditions, the mTORC1 complex is inhibited and Ulk1 is dephosphorylated, which then allows Ulk1 to phosphorylate both Atg13 and FAK family kinase-interacting protein of 200 kDa (FIP200), thus initiating nucleation [189]. Nucleation also requires the presence of a phosphoinositide 3-kinase (PI3K) complex, comprising vacuolar protein sorting 34 (Vps34), Beclin1 (Bcl1), p150, autophagy and Bcl1 regulator 1 (AMBRA1), Atg14, UV resistance-associated gene (UVRAG) and Bif1, which is essential for nucleation of the phagophore.

Ulk1, when activated, phosphorylates both Bcl1 and AMBRA1, activating them both in the process. Bcl1 increases the affinity of Vps34s for binding to other members of the PI3K complex, enhancing Vps34s catalytic activity to convert phosphatidylinositol (PI) to phosphatidyl inositol triphosphate (PI3P). PI3P is essential for phagophore elongation and recruitment of a range of other autophagy proteins to the phagophore membrane [186, 190, 191]. It is still debated what the source of the phagophore membrane is; however, there have been strong indications that the membrane is largely acquired from the endoplasmic reticulum (ER) [192]; other sources include the plasma membrane [193], the mitochondria [194] and the ER-Golgi apparatus intermediate compartment [195].

1.3.2 Membrane elongation and nucleation

Elongation of the phagophore membrane involves two ubiquitin-like reactions, the first reaction involves the binding of Atg5 to Atg12; Atg7 and Atg10 are part of the intermediate steps, to form Atg5-Atg12, and this pairing then binds with Atg16L to form an Atg12-Atg5-Atg16L complex. This complex associates with the extending phagophore membrane, inducing its curvature by recruiting microtubule-associated protein light chain 3 (LC3)-II to the membrane in an asymmetric manner [186, 190, 196]. The second ubiquitin-like reaction involves LC3; this protein is cleaved by Atg4 upon induction of autophagy, to form LC3-I. LC3-I is then activated by Atg7 and Atg3, phosphatidylethanolamine (PE) then binds to form LC3-II. LC3-II is recruited to the phagophore membrane in an Atg5-Atg12 dependent manner. p62 protein (also called sequestesome 1 (SQSTM1) is involved in selecting the cargo to be degraded, by binding to ubiquitinated proteins and moving them to the nucleating phagophore where they bind to LC3-II (Figure 1.4) [186, 190].

1.3.3 Autophagosome maturation and lysosomal fusion

Once the extending phagophore fully encapsulates the cargo, forming the autophagosome, the Atg12-Atg5-Atg16L complexes on the outside of the membrane dissociate. The autophagosome then migrates to lysosomes, in the perinuclear region of the cell, via microtubule and dynein-dynactin motor complex dependent transport [197], the autophagosome then fuses with the lysosome to form

an autophagolysosome. The fusion step of autophagy involves the formation of one of two complexes containing soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. This process is performed by either of two SNARE complexes, STX7-SNAP29-YKT6 [198] and STX-17-SNAP29-VAMP7/VAMP8 [199, 200]. Several tethering factors are required to hold both the autophagosome and lysosome close together for them to fuse; tethering factors include PLEKHM1 [201], the HOPS complex [202] and EPG5 [203]. Upon fusion, more than 60 proteases in the lysosome are involved in degradation of the autophagosome cargo [204] and are extremely susceptible to pH changes [205]. Once the cargo is degraded, the catabolites are exported back in to the cell via several transporters on the lysosomal membrane [206]; however, this process is not well understood.

1.3.4 Autophagy and inflammation

Autophagy plays a central role in the differentiation, maintenance and function of various immune cells to ensure appropriate immune responses; the immune cells include neutrophils [207, 208], monocytes and macrophages [209-211], dendritic cells [212], natural killer (NK) cells [213], T cells [214] and B cells [215]. This indicates that dysregulation of this essential mechanism will have a profound impact on the immune system.

Dysregulated autophagy underlies the pathogenesis of several autoinflammatory diseases. As autophagy plays a vital role in regulation of the NLRP3 inflammasome, mutations that upregulate the activation of the NLRP3 inflammasome can overwhelm the homeostatic mechanisms, leading to autoinflammation [216]. Not only does autophagy prevent activation of the NLRP3 inflammasome, via clearance of its components and removal of the activated NLRP3 inflammasome from the cell and pro-IL-1β [217-219], but also plays an essential role in the removal of NLRP3 inflammasome activators, such as mtDNA [220], ROS [221, 222], high-mobility group box protein 1 DNA [223], and β-amyloid plaques [224]. In addition to this, the inhibition of autophagy results in an increase in NLRP3 activation [225], leading to the conclusion that autophagy is central to the regulation of the NLRP3 inflammasome and prevention of inflammation.

Other autophagic mechanisms directly involve individual autophagy proteins. Accumulation of the autophagy protein, p62, has been shown to activate the p65 subunit of NF-κB and its translocation to the nucleus, thus increasing activation of the NLRP3 inflammasome as well as production of pro-inflammatory cytokines, IL-1β and IL-6 [226]. Atg5, on its own, helps to protect against *Mycobacterium tuberculosis* (*M. tuberculosis*) infections, mediates neutrophil apoptosis and reduces lung inflammation [227]. Various studies have shown that loss of individual autophagy proteins, such as Atg16L1, Atg7 and Atg5, results in excessive IL-1β production with increased frequency of infection by various pathogens, and the induction of pyroptosis [228-230]. Furthermore, deficiencies in LC3 and Beclin1 lead to increased caspase-1 activation, IL-1β and IL-18 secretion [231].

Complicating this story, however, are reports that inhibition of autophagy reduces IL-1β secretion in neutrophils, while another study showed that inhibition of autophagy in macrophages brought about an increase in IL-18 production but decreased production of TNF, IL-6 and IL-8 [219], indicating that autophagy acts either to reduce cytokine secretion or increase it, depending on the prevailing cellular context [232]. For example, the autophagy inducer, rapamycin, has been shown to polarise macrophages to an M1 phenotype, which shows that, in some contexts, autophagy can be a pro-inflammatory mechanism [233].

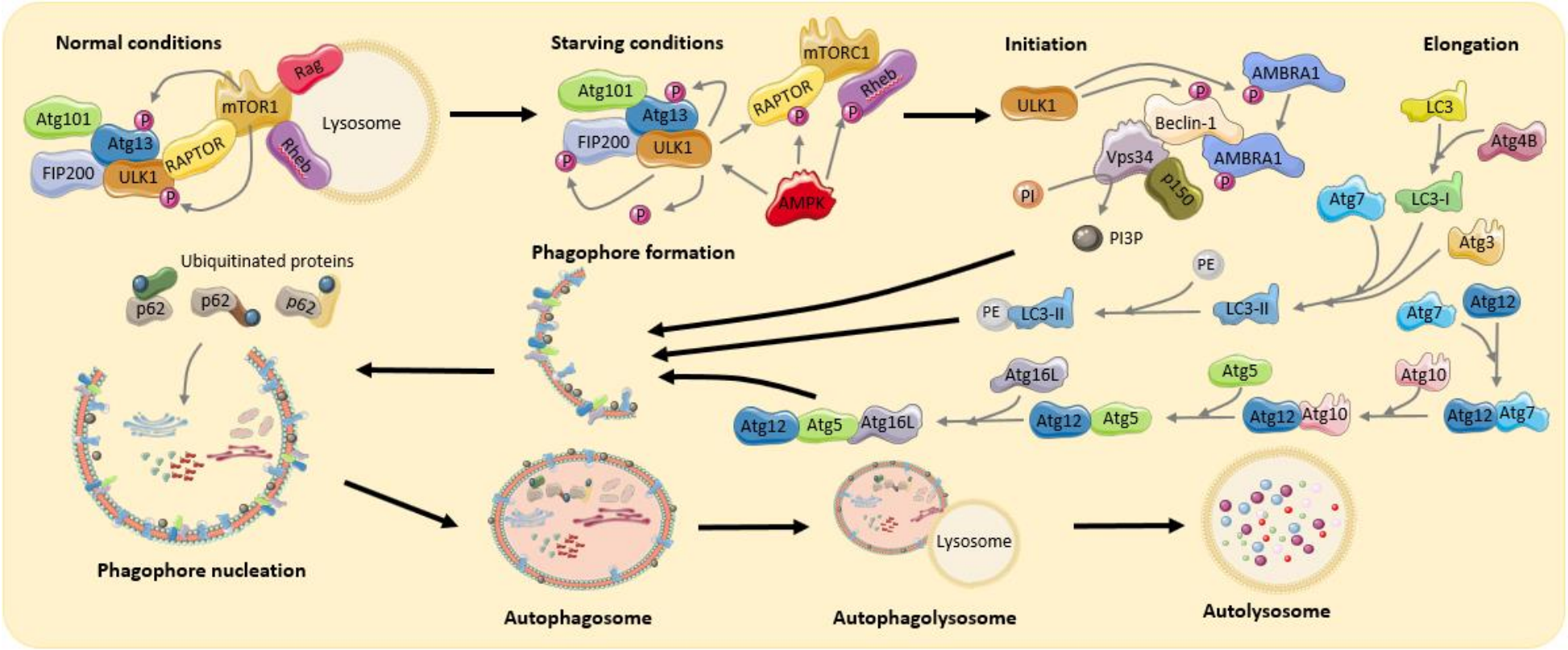


Figure 1.4 - Main autophagy machinery. The molecular pathway of the core autophagy protein components is shown, starting from normal conditions, to the initiation (under starvation conditions), with formation, elongation and nucleation of the phagophore, through to the fusion of the autophagosome and onto the lysosome to form the autolysosome. Several autophagy proteins have been left out, for simplification. Adapted from Glick *et al.* 2010 [186] and Ravanan *et al.* 2017 [190]. Figure produced using Smart Servier Medical Art.

1.5 Mitophagy

Mitochondria are vital dynamic organelles, acting as the powerhouse of the cell and immunity, as well as regulating cellular energy and cell death [234-236]. To maintain their quality, mitochondria are constantly undergoing fusion and fission [237]. Accumulation of mtDNA mutations, as well as the decline in mitochondrial activity, is believed to contribute to the normal ageing process [238].

Mitophagy is the selective degradation of damaged, dysfunctional or old mitochondria, via the autophagic pathway, acting as an essential quality control mechanism for mitochondria. Dysregulation of this pathway has been implicated in several metabolic, neurodegenerative, and autoinflammatory diseases. There are many other forms of selective autophagy, but these are outside the scope of this thesis; briefly, these forms of selective autophagy include pexophagy (removal of peroxisomes) [239], er-phagy (the ER) [240], ribophagy (ribosomes) [241], lipophagy (lipid droplets) [242], and xenophagy (invading organisms) [243].

Mitophagy can be split in to two different pathways, namely the PINK1/Parkin-mediated mitophagy (canonical) pathway and the receptor-mediated mitophagy (non-canonical) pathway; although both of these types of pathway have been well studied, it remains unknown as to how these two types of mitophagy differ in their expression across different tissues and cells (Figure 1.5) [244].

1.5.1 Canonical Pathway

Under normal conditions, when the mitochondrial membrane is polarised, the serine/threonine kinase, PINK1, moves to the mitochondrial outer membrane (OMM) and is translocated across to the inner mitochondrial membrane (IMM), where it is degraded by mitochondrial protease enzymes. However, when mitochondria become damaged, the mitochondrial membrane is depolarised, leading to accumulation of PINK1 on the OMM (Figure 1.5) [245]. The E3 ubiquitin ligase, Parkin, is recruited by PINK1 on the OMM, phosphorylating Parkin at Ser65 and activating it; PINK1 also phosphorylates ubiquitin at Ser65 which also activates Parkin [246-248]. The

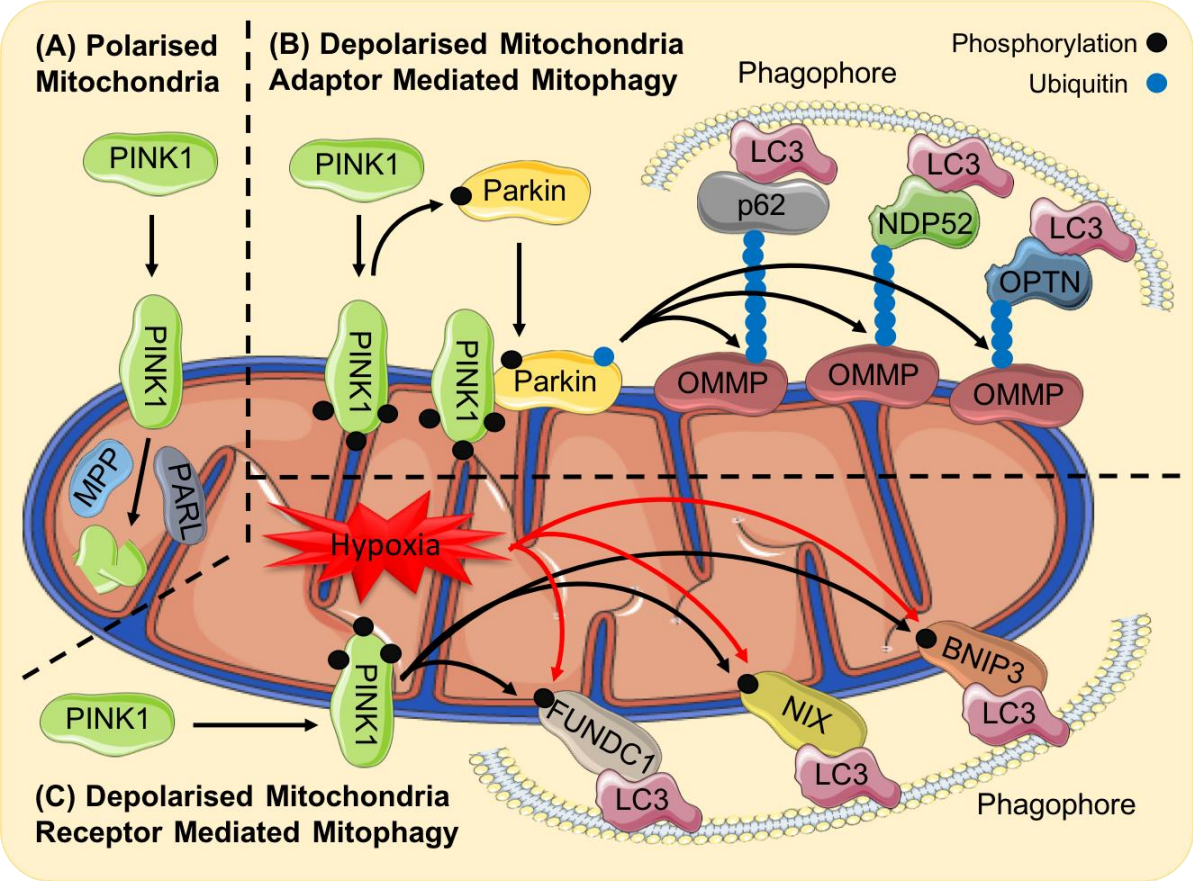


Figure 1.5 – Autophagic degradation of mitochondria (mitophagy). (A) Under normal conditions PINK1 migrates to the mitochondria where it fuses with the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM), where it migrates into the mitochondria to be degraded by mitochondrial proteases. (B) Once mitochondria become damaged/depolarised, PINK1 moves to the OMM where it resides and is phosphorylated, it phosphorylated PINK1 then phosphorylates Parkin, causing it to bind and form a complex with PINK1 on the OMM. Parkin is the ubiquitinated, and then goes on to ubiquitinate other OMM proteins. Adaptor proteins on an expanding phagophore, such as p62, NDP52 and OPTN, bind to the ubiquitin chains and target the mitochondria for autophagic degradation. (C) Receptor mediated mitophagy occurs in a similar way to adaptor mediated mitophagy, however Parkin is not involved, and mitochondrial membrane proteins are phosphorylated by phosphorylated PINK1, and then directly bind to LC3 on an expanding phagophore to undergo autophagic degradation. Figure produced using Smart Servier Medical Art.

polyubiquitination of Mfn 1 and 2, triggers their removal via the proteasome, and this leads to the prevention of mitochondrial fusion and isolation of the damaged mitochondria, thereby reducing the size of the mitochondria, making it easier to sequester them in autophagosomes with subsequent removal [249, 250].

Parkin initiates ubiquitin binding to its substrates via K48 and K63 linkages; this ubiquitination step acts as a signal to the autophagic machinery to remove the mitochondria leading to p62 binding to the ubiquitin on the OMM, via its ubiquitin binding domain. Once bound, p62 interacts with LC3 on

an expanding phagophore via its LC3-interacting motif, the mitochondria is encapsulated in the expanding phagophore, facilitating the degradation of the mitochondria [251, 252].

There are several other ubiquitin binding proteins with an LC3-interacting motif involved in mitochondrial degradation, other than p62; these include NBR1 [253], NDP52 (also known as CALCOCO2) [254], TAX1BP1 [255] and optineurin (OPTN) [256-258]. OPTN is among the most studied of the mitochondrial LC3 recruitment proteins, via the PINK1/Parkin pathway [258]. Once OPTN recruits TBK1, both OPTN and NDP52 are phosphorylated by TBK1, further promoting mitophagy [259, 260].

1.5.2 Non-canonical pathway

There are several other mitophagy pathways which do not involve the PINK1-Parkin pathway. The non-canonical pathway involves direct interaction between mitochondrial membrane proteins, such as FUN14 domain containing 1 (FUNDC1) [261], Bcl2/adenovirus E1B 19kDa interacting protein (BNIP3) [262], NIP3-like protein X (NIX/BNIP3) [263, 264] and LC3 on the expanding phagophore; these receptors are found on the OMM (Figure 5). BNIP3 was initially found to be involved in cell death but was later shown to contain a LIR domain and to be involved in mitophagy, where, upon binding of BNIP3 to LC3, BNIP3 is phosphorylated at sites Ser17 and Ser24, inducing mitochondrial engulfment by an expanding phagophore [262, 265, 266]. Cells that are deficient in BNIP3 have been shown to induce the cleavage of PINK1 and, therefore, the inhibition of mitophagy, whereas in normal cells under hypoxic conditions, BNIP3 is upregulated and expressed on the OMM, inhibiting the cleavage of PINK1, and the stimulation of mitophagy [267]. BNIP3 has been shown to interfere with mitochondrial dynamics, as well as the disassembly of OPA1, promoting mitochondrial fission, and recruitment of Drp1 to the mitochondria [268, 269].

Another mitophagy OMM protein, FUNDC1, is upregulated on the OMM and binds to LC3, under hypoxic conditions, whereas under normal conditions FUNDC1 is phosphorylated at sites Ser13 and Tyr18 by CK2 and Src, respectively [261]. When receptor-mediated mitophagy is induced,

phosphoglycerate mutase family member 5 (PGAM5) is activated and proceeds to dephosphorylate FUNDC1, leading to its binding to LC3 [270]. There are multiple mechanisms to fine tune FUNDC1 mediated mitophagy, such as via the mitochondrial E3 ligase MARCH5 and RIPK1, which inhibit FUNDC1-mediated mitophagy and induce apoptosis in the presence of cardiac injury [271, 272]. Recently, Ulk1 has been reported to play a role in mitophagy, via its phosphorylation of FUNDC1 at site Ser17, whereby it induces mitophagy under stress conditions [273].

NIX also acts as a receptor for receptor-mediated mitophagy, via its direct interaction with LC3, and is crucial in preventing the accumulation of damaged mitochondria, apoptosis and deficient cellular differentiation [263, 274]. Again, under hypoxic conditions and regulated by HIF1α, NIX is phosphorylated at site Ser81 to induce mitophagy [275, 276]. NIX also retains a specialised motif, located on the cytoplasmic domain in the mitochondria, which signals to upregulate other mitophagic proteins [277]. Other more subtle regulations of mitophagy include the phosphorylation of NIX at residues Ser34 and Ser35, which are located close to the LIR domain, and this increases the binding affinity of NIX to LC3 [278]. Studies using *C. elegans* have also shown that DCT-1, the mammalian homologue for both BNIP3 and NIX, maintains cellular homeostasis during aging [279].

The mechanisms by which BNIP3, FUNDC1 and NIX interact and modulate mitophagy are still ill defined, further research to characterise the mechanisms will be highly interesting. However, there have been some hints as to how NIX, FUNDC1 and BNIP3-mediated mitophagy interact, as one study showed the involvement of Rheb GTPase in the mitophagic process [280].

There are other mitochondrial receptors, such as Prohibitin 2 (PHB2) [281] and cardiolipin [282], involved in mitophagy, however, they are located on the inner mitochondrial membrane (IMM). Upon Parkin-dependent disruption of the OMM, the IMM mitophagy receptors, PHB2 and cardiolipin, become exposed to the cytosol and are ultimately bound by LC3BII on an expanding phagophore [283, 284]. Unlike the other mitophagy receptors, cardiolipin is a phospholipid, which is synthesised and located on the IMM; once the OMM is damaged, along with PHB2, cardiolipin moves to the OMM to

interact with LC3BII [282]. Recently it has been shown in yeast that mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) are involved in cardiolipin mediated mitophagy [284].

1.5.3 Mitophagy and metabolism

Mitophagy contributes to metabolic changes during differentiation of cells, such as adipocytes, when they are transitioning from beige to white adipocytes [285]. Hypoxia has also been shown to induce a switch from oxidative phosphorylation to glycolysis which has been indicated to increase HIF1α, resulting in elevated NIX-dependent mitophagy during differentiation of retinal ganglion cells (RGCs). During macrophage differentiation NIX-dependent mitophagy has also been suggested to contribute towards an M1 pro-inflammatory and more glycolytic phenotype, but it does not contribute to an M2 phenotype, as M2 macrophages rely on oxidative phosphorylation [285]. Inhibition of autophagy and mitophagy may result in increased mitochondrial mass and decreased expression of glycolytic genes [285].

Inhibition of oxidative phosphorylation prevents repolarisation of macrophages from M1 to M2 and inhibition of nitric oxide production, an important effector molecule in the polarisation of M1 macrophages, which results in a reduced rate of mitochondrial functional decline, improving metabolism and, thus, repolarisation to M2 macrophages. This indicates that an improvement in mitochondrial quality can lead to a switch from glycolysis, and a M1 macrophage pro-inflammatory phenotype, to oxidative phosphorylation and a more M2 macrophage anti-inflammatory phenotype [286].

PINK1 deficiency has been linked to the significantly decreased ability of induced pluripotent stem cells (iPSCs) to reprogram and has been shown to spontaneously differentiate and form heterogenous cell populations. PINK1-deficient IPSCs also present with considerable alterations in various glycolytic and tricarboxylic acid cycle (TCA) related metabolites, such as α-ketoglutarate, an important suppressor of cell differentiation in stem cells [287]. During cellular differentiation mitochondria become long and tubular in shape, with an increased mass and metabolically switch to

oxidative phosphorylation, thereby providing further evidence of the importance of mitochondria in cellular differentiation [288].

1.5.4 Metabolism in CF

Although mitochondrial defects have been reported in CF, as far back as 1979, the state of mitochondria and metabolism has not been investigated in any great detail. The first mitochondrial defect to be found was in skin fibroblast cells from CF patients; these cells had a higher oxygen consumption rate (OCR) with mitochondrial Complex I (mCx-I) found to be more sensitive to inhibition by rotenone compared to HC cells [289]. The same group was also able to demonstrate, in skin fibroblast cells, that the optimal pH and Km values are altered for NADH dehydrogenase [290], and that Ca^{2+} uptake is raised, which is likely to be related to altered respiratory system activity [291]. However, in 2009, another group found that Ca^{2+} uptake into mitochondria was downregulated in homozygous F508del-CFTR airway epithelial cells compared to non-CF cells, and that the mitochondria were fragmented and mitochondrial membrane potential decreased; on the other hand, the differences in results between the two groups could be due to the different cell types used [292]. Furthermore, nasal epithelial cells, from CF patients, presented with increased OCR and heightened superoxide (O_2^-) and peroxide (H_2O_2) production [293, 294]. After the Feigal group's results in 1982, it was suggested that the gene responsible for causing CF might be part of mCx-I; however, this hypothesis was dismissed after the CFTR was found to be a Cl^- ion epithelial transport protein [290]. After this, interest in the mitochondria in CF decreased, with only a few more publications reporting that mitochondrial proteins appeared abnormal through 2D-electrophoresis analysis [295] and that there was increased activity of the four glycolytic enzymes hexokinase, phosphofructokinase, pyruvate kinase and lactate dehydrogenase in skin fibroblasts [296], and, in addition, the intracellular pH was raised upon workload in skeletal muscle in CF [297].

In more recent years, interest in the mitochondria in CF has begun to increase, with findings such as the CFTR regulation of the expression of certain mitochondrial proteins, such as the CFTR

dependent upregulation of the outer mitochondrial membrane protein CDGSH iron sulfur domain 1 (CISD1), which transports iron into the mitochondria [298], and mitochondrial NADH-ubiquinone oxidoreductase chain 4 (MTND4), which is a subunit of mCX-I [299]. The inhibition of CFTR has been proposed to reduce mCx-I activity in several epithelial cell lines [300]; however, the inhibitors used had been shown to alter the mitochondria in the absence of a functioning CFTR protein, indicating that it was these inhibitors' off target affects that altered the mitochondria [301]. As mCx-I and mCx-III are responsible for the majority of leakage of electrons from the electron transport chain (ETC) and, therefore, for the production of mROS, as first suggested in 1992 [302], the dysregulation of mCx-I in CF leads to increased mROS production [79].

The phosphatase and tensin homolog (PTEN) is a regulator of metabolism and cell proliferation, and under normal conditions it forms a complex with the CFTR at the plasma membrane; however, in CF, PTEN is unable to form a complex with the CFTR, leading to mitochondrial dysfunction with increased mROS and succinate release, promoting colonisation of the lungs with *P. aeruginosa* [303]. This is backed up by other studies, in CFTR KO mice and CF HBEC lines, showing that oxidative stress is prevalent, with increased oxidation of mtDNA, ROS production and reduced mitochondrial GSH [304]. The addition of GSH monoethyl ester increased the mitochondrial GSH levels in CFTR KO mice and CF HBEC lines, and led to increased mCx-I activity with recovery of mitochondrial membrane potential, showing the importance of the antioxidant balance between GSH and GSSG, and its effect on mitochondrial function [305]. However, the nature of the link between abnormal mCx-I function and GSH levels, as well as which is altered first, is still unknown. Clinical trials, using GSH administration as a treatment, have thus far proven only mildly effective or not at all [306-308]. Using the CF HBEC lines, CFBE41o and the WT equivalent, Reshkin *et al* were able to characterise mitochondrial function in CF, with increased mROS production as well as abnormal mitochondrial membrane potential, OCR, mCx-I and IV, in addition to adenine nucleotide translocator-dependent ADP/ATP exchange. The use of the CFTR modulators, lumacaftor and 4,6,4'-trimethylangelicin (TMA) was able to improve these abnormalities to values closer to that of the WT [309], demonstrating that the mitochondrial defects

found in CF are primarily caused by the dysfunction in Cl^- activity. The metabolomic profile of human airway epithelial cells, taken from patients with CF, showed a unique profile compared to that of HC participants [310], and glucose levels in the ASL and oxidative stress in epithelial cells were shown to be modulated by glucose-related metabolic pathways [311].

1.5.5 Mitochondria and inflammation

The mitochondria in recent years have been shown to be key regulators of inflammation via the NLRP3 inflammasome [312]. Given that many autoinflammatory diseases are driven by recurrent sterile inflammation through NLRP3 inflammasome activation [313, 314], it stands to reason the mitochondria play an important role in CF. Recent publications have linked a dysregulated UPR response in CF monocytes and M1 macrophages to an altered metabolic state and hyperinflammation through the IRE1α arm of the UPR, further demonstrating the mitochondria's role in the pathogenesis seen in CF [103].

Mitochondria can act as a scaffold for NLRP3 inflammasome activation, this process is mediated by cardiolipin, where, upon depolarisation of mitochondria, cardiolipin moves to the OMM, where it acts as a scaffold for NLRP3 inflammasome formation [315]. Another protein, mitochondrial antiviral signalling (MAVS) protein, which is involved in retinoic acid-inducible gene (RIG)-I-mediated IFN responses to viral infections, also acts as a platform for NLRP3 inflammasome activation [316]. The positioning of NLRP3 on the mitochondria is thought to enable the immediate recognition of mitochondrial damage, emphasising the importance of the link between detecting mitochondrial PAMPs and NLRP3 inflammasome activation.

Another recent insight into the role mitochondria play in regulation of the NLRP3 inflammasome is the discovery of mitochondrial micropeptide-47 (Mm47), located on the mitochondria. siRNA knockdown of Mm47, as well as KO of Mm47 with CRISPR-Cas9, are both associated with a deficient NLRP3 but normal NLRC4 and AIM2 responses, underlining the importance of Mm47 in activation of the NLRP3 inflammasome [317].

A prominent role of Ca^{2+} mobilisation has also been demonstrated in activation of the NLRP3 inflammasome, via the promotion of mitochondrial damage [318, 319]. Binding of ATP to the transmembrane ionotropic receptor, P2X purinoceptor 7 (P2RX7), increases intracellular Ca^{2+} ion levels, leading to damaged mitochondria [320]. The intracellular Ca^{2+} ion concentration is closely linked to mitochondrial activity, with increased intracellular Ca^{2+} ions leading to increased metabolic activity; however, a significant increase in Ca^{2+} ions can rupture mitochondrial membranes [321, 322]. The highest concentration of Ca^{2+} ions is found in the ER, and comparatively the cytosol has relatively low levels; upon activation of the ER Ca^{2+} ion channel, 1, 4, 5-triphosphate-receptor (IP3R), Ca^{2+} are released into the cytosol and can be taken up by the mitochondria [321, 323]. This efflux of Ca^{2+} from the ER can occur in a much more regulated manner, via the mitochondria-associated membrane (MAM); this is the name given to the contact site between the ER and mitochondria, which allows Ca^{2+} ion release to be more closely regulated to influence the metabolism [324]. The release of Ca^{2+} ions would have to be near mitochondria for this to have an effect, this is due to Ca^{2+} ions being quickly chelated in the cytosol [323].

Monosodium urate (MSU) crystals can indirectly result in damaged mitochondria, as MSU crystals are enclosed by lysosomes eventually leading to their rupture. Lysosomal proteases, such as Cathepsin B, are activated and released into the cytoplasm due to lysosomal rupture and this leads to the mitochondrial membrane becoming damaged and eventually depolarised, releasing mitochondrial contents and causing NLRP3 inflammasome activation, indicating that lysosomal damage is a DAMP for NLRP3 activation [325, 326].

MROS is another activator of the NLRP3 inflammasome; given that mitochondria produce mROS under normal conditions, as a by-product of respiration, only an increased production of mROS is considered to be an inflammasome activator, as the mitochondria is likely to be damaged or have an increased metabolic state [327]. The inhibition of mROS release has been shown to reduce NLRP3 driven inflammation in both MKD and TRAPS [164, 171].

There are a number of feedback loops involving the NLRP3 inflammasome and mitochondria, which either lead to enhanced activation of the inflammasome or inhibition of its activation; for example, NLRP3 activation can lead to mitochondrial damage and inhibition of mitophagy through caspase-1 cleavage of Parkin [328], further activating the inflammasome. Dysregulated mitophagy may also cause excessive NLRP3 activation in MKD, leading to accumulation of damaged mitochondria and increased IL-1β secretion [164, 329]. Loss of function of some autophagy proteins has been shown to result in the accumulation of abnormal mitochondria and translocation of mtDNA to the cytosol, in response to LPS and ATP stimulation, contributing to NLRP3 inflammasome activation [231].

1.6 Defective autophagy in CF

In CF the accumulation of substantial quantities of ΔF508 CFTR, has been reported to result in defective autophagy, which was first demonstrated in 2010 in HBEC lines by Luciani *et al*. [79]; subsequently, this has also been shown to occur in bone marrow derived macrophages (BMDMs) from a mouse model [330]. This significant protein accumulation amounts to ER stress and a pro-oxidative environment, with excessive amounts of ROS being produced. This, in turn, leads to the small ubiquitin-like modifier (SUMO)ylation of transglutaminase 2 (TG2), which inhibits the degradation of TG2 (Figure 1.6). This occurs due to overactivation of protein inhibitor of activated STAT (PIAS)γ, by increased levels of ROS. PIASγ regulates the SUMOylation of TG2 in a cascade reaction involving E3 ligases, which ultimately inhibits the ubiquitination of TG2, thus sustaining high levels of TG2 [331]. These deleterious effects on TG2s post translational modification activity have also been revealed by inhibition and gene silencing of the CFTR, in WT CFTR cell lines [331]. In healthy cells, TG2 has a range of important functions, acting as a regulator of the post-translational network and protein degradation balance. TG2 is regulated by several factors, such as Ca^{2+}, NO, ROS, and nucleotides. In a high Ca^{2+} environment, TG2 creates covalent bonds (cross-linkages) on targeted proteins, as a post-translational modification, whereas in low Ca^{2+} concentrations it can function as a G-protein or a protein disulphide isomerase [332]. In CF however, these post-translational regulatory mechanisms

are distorted. TG2, therefore, plays a key role in the impairment of autophagy, by creating cross-linkages between several different autophagy proteins, such as Bcl1, Vps34, AMBRA1, which are key components of the initiation step of autophagy. This results in their removal from the ER membrane and accumulation, along with the CFTR and the anti-inflammatory peroxisome proliferator-activated receptor (PPAR)γ, in p62 positive aggresomes [79]. Defective autophagy has deleterious knock-on effects, with the proteasome becoming overloaded, as it is unable to clear all the misfolded protein that autophagy would normally clear. This exacerbates the degree of cellular stress, bacterial clearance impairment, and results in exaggerated inflammation [79, 333].

Several autophagy genes have reduced expression in CF cells [330, 334]; although, the ubiquitin binding protein p62 has been shown to be raised [79]. The p62 protein accumulates under conditions where autophagy is defective and binds to the protein aggregates. The knockdown of p62, via gene silencing, however, results in a reduction in these protein aggregates as well as improving CFTR maturation and trafficking to the membrane, indicating p62s role in CF pathogenesis [79]. This all occurs even though the faulty CFTR induces a pro-autophagy state, as there is a decreased association of Bcl1 with its key regulator, Beclin2 (Bcl2), indicating a pro-autophagy environment [79].

It is well established that macrophages in CF have an impaired ability to remove bacterial infections, and that, in healthy cells, the CFTR is expressed on the macrophage membrane, as well as lysosomal and phagosomal membranes [73, 115, 335, 336]. More recently, a study of the DNA methylation profile of CF macrophages showed that the promoter regions of Atg12 are significantly more methylated than WT controls, resulting in decreased protein expression of Atg12 and compromised autophagy [337].

The depletion of p62 has been linked to an increase in *B. cepacia* complex clearance in CF macrophages, whereas the overexpression of p62 results in increased *B. cepacia* complex survival. The reduction in p62 has also been shown to release Bcl1 from the CFTR aggresomes and increase its recruitment to the phagophore, thereby improving bacterial clearance [334]. The mTOR inhibitor, rapamycin, also inhibits *B. cepacia* complex infection and reduces lung inflammation [330].

1.7 Mitophagy in CF

So far, no research has been done as to whether mitophagy is functioning normally in CF. With the obvious links between mitochondria, NLRP3-driven inflammation and dysregulated autophagy, it stands to reason there are likely to be abnormalities in the selective degradation of mitochondria. Another question worth investigating is, if mitophagy is dysregulated in CF, whether this leads to an accumulation of mitochondria that are more damaged or more prone to being damaged, and whether this results in altered metabolism and heightened inflammation.

1.8 Targeting autophagy as a treatment

A new combinational therapy of cysteamine, a re-purposed drug originally used to treat cystinosis [338], and EGCG, the most common flavonoid in green tea, has recently been studied and shows promising results. These effects have been shown, both *in vitro* and *in vivo*, in murine and human studies, in subjects homozygous for the *ΔF508* mutation or *ΔF508/null-CFTR* [79, 339-341]; however, this treatment seems to be ineffective in patients with more rare mutations [339].

Cysteamine, originally used for the treatment of cystinosis, where it is administered to prevent intralysosomal cysteine accumulation, has been considered for a number of different applications, including non-alcohol fatty liver disease and neurodegenerative diseases [342]. Cysteamine has been shown to improve Bcl1 expression and restore autophagy in CF [79, 341], through inhibition of TG2 (Figure 1.6). It has also been demonstrated to act as a mucolytic agent, breaking down the thick mucus in the CF lung and disrupting biofilm formation, as well as having bactericidal properties against *P. aeruginosa* [343, 344]. Recently, the inability of macrophages to effectively internalise and clear opportunistic infections from pathogens, such as *P. aeruginosa, S. aureus* and *B. cepacia* complex, has been described, and can be directly linked to the defective CFTR ion channel and autophagy. The restoration of autophagy in macrophages, by use of cysteamine, a proteostasis regulator, can restore the macrophages' ability to internalise and clear *P. aeruginosa* and reduce TNF production [345]. Cysteamine has also been shown to improve clearance of antibiotic-resistant bacteria in CF patients'

peripheral blood monocyte-derived macrophages and alveolar macrophages [346], as well as improving the expression of a partially functional CFTR on CF patients' HBECs [79, 341]. A cysteamine conjugate has also been tested with Orkambi and found to have an additive effect in improving chloride efflux [347].

EGCG has been shown to inhibit CK2, an enzyme that regulates a number of targets, such as NF-κB [348, 349] (Figure 1.6), JAK/STAT pathway [350], PI3K/Akt pathway [351] in a range of cells. EGCG has also been shown to prevent fragmentation and degradation of the CFTR through inhibition of CK2 [53, 340], as CK2 phosphorylates the CFTR at position T1471, decreasing its stability and promoting its fragmentation [53]; this results in improved expression of the CFTR on the cell membrane. EGCG has also been reported to reduce the methylation of Atg12 in CF macrophages, as well as improving autophagy and restricting B. *cepacia* replication in CF mice [337].

Another small molecule, amiodarone, a Ca^{2+} channel blocker, has been found to complement cysteamine in improving autophagic function and promoting expression of ΔF508 CFTR on the cell membrane, although the mechanism by which amiodarone achieves this is unclear [352]. Furthermore, autophagy and proteostasis regulators are well covered in the recent review by Bodas *et al* 2019 [353].

In combination, cysteamine and EGCG have been shown to reduce inflammatory cytokine levels in CF patients' sputum, with improved CFTR expression in patients' nasal epithelial cells as well as improving lung function and reducing the amount of Cl^- secreted in the sweat test to normal levels [339, 340]. These data indicate their potential as an affordable therapy for patients with CF.

The recent development of CFTR small molecule-based therapies provides an opportunity to investigate the impact CFTR modulators have on the function of autophagy in CF.

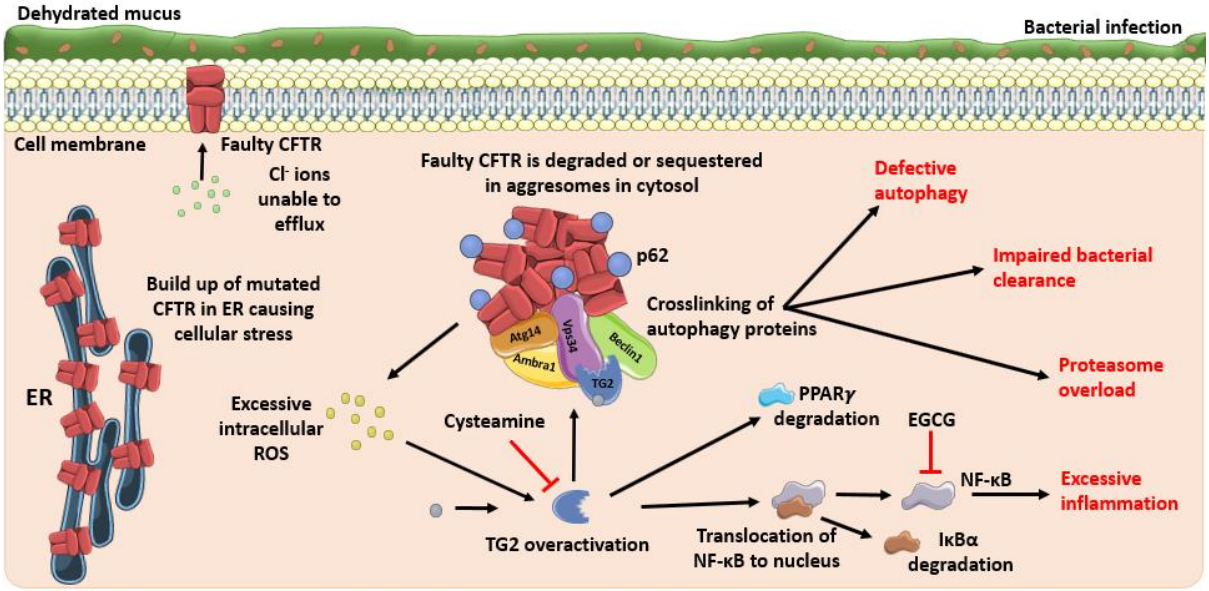


Figure 1.6 – Defective autophagy in cystic fibrosis. Improper folding of the CFTR due to mutation leads to retention of CFTR within the ER, triggering cellular stress. The CFTR that does leave the ER is fragmented and degraded, however the proteasome becomes overloaded and the CFTR becomes sequestered in aggresomes within the cytosol. The increase in intracellular ROS due to the heightened cellular stress results in the increase in SUMO E3-ligase PIASy, leading to the SUMOylation and inhibition of TG2 ubiquitination; therefore, a high concentration of TG2 is retained within the cell. This increased level of TG2 mediates the formation of covalent bonds, between several autophagy proteins, such as Beclin1. This causes them to move away from the ER membrane to be retained in aggresomes in the cytosol, inhibiting autophagy. The clearance of ROS producing mitochondria by autophagy is thus impaired, perpetuating the cycle. TG2 also creates cross-linkages between PPARγ and IκBα, promoting their ubiquitination and hence proteasome degradation, favouring the inflammatory NF-κB translocation to the nucleus instead. Cysteamine and epigallocatechin gallate (EGCG) are used here to inhibit TG2 and NF-κB respectively. Adapted from Villella *et al* [354]. Figure produced using Smart Servier Medical Art.

1.9 Hypotheses

The hypotheses shown below were established, based on the most recently published literature as well as clinical observations made at the Cystic Fibrosis Adult Ward, St. James's Hospital, Leeds, UK.

The overarching hypothesis for this thesis is that autophagy is dysregulated in CF cells homozygous for ΔF508 CFTR, which will lead to an accumulation of damaged/damage prone mitochondria, abnormal metabolism, and will contribute to the inflammatory state in CF.

1.9.1 List of Hypotheses

Hypothesis 1: That the autophagy-lysosome pathway is in some way dysregulated in CF as a result of defective CFTR expression and function.

Hypothesis 2: That autophagy will be partially improved by using CFTR modulators.

Hypothesis 3: That the clearance of mitochondria by autophagy is dysregulated, and leads to an increased number of mitochondria that are damaged, or more prone to damage, as well as altered metabolism in CF.

Hypothesis 4: That the use of cysteamine and EGCG may recover dysregulated autophagy and reduce the hyperinflammatory state found in CF.

1.9.2 Aims and Objectives

Hypothesis 1:

1 – To investigate the expression of autophagy genes and the turnover of autophagy proteins in HBECs, both with and without CF mutations, as well as in monocytes and M1 macrophages from patients with ΔF508 homozygous mutations, in comparison with HCs.

2 – To establish the number of autophagosomes being produced and turned over in CF and HC cells.

3 – To clarify whether the quantity or quality of lysosomes found in CF differs from HC cells, and if this contributes to dysregulated autophagy in CF.

Hypothesis 2:

1 – To investigate whether CFTR modulators affect the expression of autophagy proteins in HBECs, both with and without CF mutations, as well as in monocytes and M1 macrophages from patients who are ΔF508 homozygous, compared to HCs.

2 – To establish whether CFTR modulators affect the number of autophagosomes being produced and turned over in CF and HC cells.

3 – To clarify whether CFTR modulators affect the quantity or function of lysosomes found in CF compared to HC cells and if this contributes to dysregulated autophagy in CF.

Hypothesis 3:

1– To investigate whether there is an increase in mitochondrial damage or mitochondria that are more prone to damage in CF.

2 – To clarify if metabolism is altered in mitochondria that are more damaged, or more prone to damage in CF.

3 – To establish whether it's the mutated CFTR's lack of function that leads to damaged mitochondria and altered metabolism.

Hypothesis 4:

1 – To investigate whether cysteamine and EGCG help to alleviate the hypersecretion of inflammatory cytokines and hyposecretion of anti-inflammatory cytokines in CF patient samples.

2 – To investigate whether cysteamine and EGCG help to prevent apoptosis through recovery of autophagy and anti-inflammatory affects.

3 – To establish if cysteamine recovers or improves autophagy in cells with a CFTR mutation.

Chapter 2.0 – Methods

2.1 Culturing of Immortalised Cell Lines

HBEC lines were grown under sterile conditions in a category two tissue culture hood. The WT cell line, Beas-2B (ATCC CRL-9609), was used as the HC cell line, as it expressed two WT CFTR channels. The cell lines used as CF models were the IB3-1 cell line (a gift from Professor Eric Blair, Leeds, UK) containing the CFTR mutations ΔF508/W1282X, CuFi-1 cell line (ATCC CRL-4013) containing the homozygous CFTR mutation ΔF508/ΔF508, and the CuFi-4 cell line (ATCC CRL-4015) containing a heterozygous CFTR mutation ΔF508/G551D. Both Beas-2B and IB3-1 cell lines were cultured in LHC basal medium (ThermoFisher Scientific) with 10% fetal bovine serum (FBS) (Gibco) and 50 IU/ml penicillin, and 50 g/ml streptomycin (1% P/S) (Sigma), CuFi-1 and 4 cell lines were cultured in LHC-9 Media (Gibco 12680013). All cell lines were cultured initially in T75 and T175 positively charged flasks (Sarstedt) at 37°C and 5% CO_2, 95% air atmosphere. Cells were left to grow until 90% confluency was reached, and then seeded in to 6 well positively charged plates (Sarstedt) at $1x10^6$ per ml per well and left overnight to allow cells to adhere to the plate.

2.2 Patient Cohort

Patients with CF were recruited from the Adult Cystic Fibrosis Unit at St James's University Hospital, Leeds. All patients were homozygous for the mutation ΔF508-CFTR and had clinical features consistent with the diagnosis of CF. All CF patients who have had a lung transplant, were on CFTR modulators or whose clinical condition was deemed unstable, were excluded from this study. Age and sex matched HCs were recruited from the Wellcome Trust Brenner Building, University of Leeds, St. James' University Hospital, Leeds, UK. Informed written consent was obtained from all study participants, HC (n=19) and CF (n=53), upon sample collection.

2.3 Processing of Patients' Blood Samples

Blood samples were collected from patients in Vacuette tubes (Greiner-bio-one) containing K_3EDTA, and the PBMCs were separated from the blood using the lymphoprep® (Axis-Shield)

technique. Whole blood was mixed with phosphate buffered saline (PBS) in equal parts, layered on top of 15 ml lymphoprep solution (Stem Cell 07861) in a 50 ml falcon tube and centrifuged at 1100 g for 20 minutes, 22°C with the brake off. The falcon tube mixture separates in to 4 layers, serum, PBMCs, lymphoprep solution and blood. PBMC buffy layer was pipetted into another falcon tube and washed with PBS twice, 1100 g, 10 minutes, 22°C with the brake on. Once the PBMCs were separated they were re-suspended in Rosswell Park Memorial Institute (RPMI) media (10% FBS, 1% P/S) and seeded in to 12 well plates (Corning Costar) at $2x10^6$ per ml and left overnight. All samples were collected and processed on the same day, with stimulations being carried out the next day. Cells were grown at 37°C, 5% CO_2, 95% atmosphere.

2.4 Monocyte isolation

To isolate monocytes from patient blood samples, the EasySep Human Monocyte Isolation Kit produced by Stem Cell Technologies (19359) was used. After PBMCs had been isolated from the blood, the PBMCs were washed in PBS without Ca^{2+} and Mg^{2+} containing 2% FBS and 1mM EDTA. The manufacturers protocol was then followed to isolate monocytes. Upon isolation, monocytes were resuspended in RPMI media (10% FBS, 1% P/S) to a concentration of $1x10^6$ per ml and seeded in to 6 well plates (Corning Costar) at $1x10^6$ cells per well. The cells were then left overnight in an incubator at 37°C, 5% CO_2, 95% atmosphere and stimulated the next day.

2.4.1 M1 macrophage differentiation

HC and CF PBMCs were collected and monocytes isolated (see above) in RPMI at 37°C, 5% CO_2, 95% atmosphere. Monocytes were stimulated to an M1 phenotype over 7 days, using GM-CSF (20ng/ml) (PeproTech EC Ltd) for the first 6 days, RPMI media was supplemented with fresh RPMI media (+50% volume) containing GM-CSF every 3 days. On the final day, the media was changed for fresh RPMI containing LPS (50ng/ml) and IFNγ (100ng/ml) (both PeproTech EC Ltd) for 24 hours.

2.5 Cell stimulations and dyes

Reagent	Concentration	Duration	Manufacturer (Mfr.)	Mfr. No.	Pathway/target
Chloroquine	50 µM	3 or 4 hours	Sigma Aldrich	C6628	Autophagosome
Tezacaftor	5 µM	48 hours	Vertex	VX-661	CFTR stability
Ivacaftor	5 µM	48 hours	Vertex	VX-770	CFTR function
ATP	5 mM	0.5 hours	InvivoGen	Tlrl-atpl	K^+ pump
LPS	10 or 50 ng/ml	4 or 24 hours	InvivoGen	tlrl-3pelps	TLR4, NFκB
ac-yvad-cmk	2µg/ml	1 hour	Sigma	SML0429	Caspase-1
Cysteamine	250 µM	24 hours	Sigma Aldrich	M9768	TG2
GM-CSF	20 ng/ml	72 hours	PeproTech	300-03	Cell Proliferation
IFNγ	100 ng/ml	24 hours	PeproTech	300-02	Macrophage activation
Mitotracker Deep Red	10 nM	0.5 hours	Invitrogen	M22426	Mitochondria
Mitotracker Green	10 nM	0.5 hours	Invitrogen	M7514	Mitochondria
MitoSOX	1 µM	0.5 hours	Invitrogen	M36008	mROS
Autophagy Assay Kit	1x as per Mfr. protocol	0.5 hours	Sigma	MAK138-1KT	Autophagosome
Autophagy Assay Kit	0.5x as per Mfr protocol	0.5 hours	Bio-Rad	APO010B	Autophagosome
Lysotracker Deep Red	50nM	0.5 hours	Invitrogen	L12492	Lysosomes
(R) BPO-27	100 nM	2 hours	MedChemExpress	HY-19778A	CFTR inhibitor

Table 2.1 – Reagents and dyes used for cells.

2.6 ELISA

Supernatants were collected from each cell stimulation and stored at -80°C. The ELISA Invitrogen CytoSet™ assay kits IL-6, TNF and IL-10 were used to analyse cytokine secretions in cell supernatant, the manufacturer's protocol was followed. Briefly, supernatants for ELISAs were defrosted on ice and centrifuged before use (5 minutes, 13,200 rpm). 96 well Nunc-Immuno ELISA plates (Thermo Scientific) were coated with the capture antibody and left overnight at 4°C. The wells were washed twice with PBS containing 0.05% Tween-20 (PBST) and tapped dry. Supernatant samples were loaded on to the plate in duplicate, along with the cytokine standards, followed by the detection antibody, and incubated for 2 hours. The contents of the plate were aspirated off and the wells washed 5 times with PBST. The anti-cytokine streptavidin-HRP conjugates were added to all wells and incubated for 1 hour, then removed by aspiration and the wells washed 5 times with PBST. The TMB substrate solution (Invitrogen) was incubated on the plate for 30 min and the reaction stopped with

sulphuric acid stop solution (1 M). The absorbance of the wells was read at 450 nm and the reference wavelength at 620 nm. The average cytokine levels were taken from the duplicate samples.

2.7 Western Blots

Cells were washed with PBS and then lysed after stimulations using radioimmunoprecipitation assay (RIPA) buffer, containing a 10% (w/v) protease and phosphatase inhibitor cocktail (Roche and Sigma respectively). Samples were centrifuged at 14,000 g for 10 min, and the protein content of the resultant supernatant was quantified using bicinchoninic acid assay. Proteins were resolved by SDS-PAGE using 10% polyacrylamide gels at 100 V for 1 hour and transferred to Immobilon P polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for 1 hour. The membrane was blocked by incubation for 1 hour at 22°C with PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) dried milk powder. The primary antibodies were incubated overnight at 4°C in PBS-Tween 20 5% (v/v) BSA and the secondary antibody was incubated for 2 hours at room temperature in 5% (w/v) milk (Table 2.2). Washes were used in between each stage using PBS Tween 20. Bound antibody was detected using enhanced chemiluminescent (ECL) detection reagent (Promega). Both Chemidoc (BioRad) and ImageLab software (BioRad) were used to analyse images obtained.

Target	Host	Dilution	Manufacturer (Mfr)	Mfr. Part No.
LC3B/MAP1LC3B	Rabbit	1/2000	Novus Biologicals	NB100-2220
p62/SQSTM1	Rabbit	1/2000	GeneTex	GTX111393
Beclin1	Rabbit	1/2000	Bethyl Laboratories	A302-566A-M
Atg5-Atg12	Rabbit	1/1000	GeneTex	GTX113309S
Vps34	Rabiit	1/1000	GeneTex	GTX129528
p150	Rabbit	1/1000	GeneTex	GTX132466S
PINK1	Rabbit	1/1000	Novus Biologicals	NB100-644
Parkin	Rabbit	1/1000	Biorbyt	Orb214359
β-actin	Rabbit	1/10,000	GeneTex	GTX124214
β-actin	Mouse	1/20,000	Sigma-Aldrich	A1978
COX4I1	Rabbit	1/1000	Cusabio Biotech	CSB-PA005832ESR1HU
TOM20	Rabbit	1/1000	Abbkine Scientific	ABP52627-2
HRP anti-rabbit	Goat	1/5000	Thermo Fisher Scientific	32260
HRP anti-mouse	Goat	1/10,000	Thermo Scientific	31430
TG2	Rabbit	1/1000	Bethyl Laboratories	A304-229A-M
LAMP1 (CD107a)	Mouse	1/1000	BD Biosciences	555798
Nrf2	Goat	1/1000	R&D Systems Inc.	AF3925-SP
KEAP1	Mouse	1/1000	Santa Cruz BioTech. Inc.	Sc-365626

OxPhos Cocktail	Mouse	1/1000	Abcam	ab110413

Table 2.2 – Antibodies for western blotting

2.8 Cathepsin D Activity Assay Kit

The kit was supplied by RayBiotech Inc. (68AT-CathD-S100) and kept at -20°C until needed. Cells were cultured as normal and then harvested using cell lysis buffer supplied in kit following manufacturers protocol. Samples were then stored at -80°C until needed. Protocol was carried out as manufacturer's instructions.

2.9 Confocal Microscopy

2.9.1 Lysotracker by Invitrogen

Collagen from human placenta (Sigma C7521) that was dissolved in 0.5 M acetic acid to a concentration of 0.1%, once the collagen was dissolved it was diluted by a factor of 10 in PBS to make 0.01% final concentration. This solution was then used to coat Merck Millicell EZ 8-well glass slides (PEZGS0816), the slides were left overnight at 37°C for collagen to attach. Collagen was then washed three times using PBS and left to dry. HBECs were seeded at 50 thousand cells per well in 500 µl of media, whereas monocytes and M1 macrophages were seeded at 100 thousand cells per well in 500 µl of media, cells were left overnight to attach and stimulated. Once stimulation had finished, Lysotracker Deep Red (ThermoFisher L12492) was added at a concentration of 50 nM and left in the incubator at 37°C for 30 minutes. Cells were then washed with PBS once and then fixed with 4% paraformaldehyde at room temperature for 15 minutes, the formaldehyde was then removed and $NaHCO_3$ (1M) was added to quench the formaldehyde. The cells were then washed 3x with PBS, the cells coated with ProLong Gold Antifade Mountant with DAPI (Invitrogen P36931) and a cover slip put on. The slide was left overnight in the dark at room temperature to dry and was imaged the next day using a confocal scanning microscope Nikon A1R, running the Nikon NIS elements analysis software.

2.10 Flow cytometry

Flow cytometry was utilised for several assays, the cytometer used was the CytoFLEX LX with Cytexpert 2.3 for analysis.

2.10.1 Monocyte Characterisation

Classical monocytes were selected through targeting of the cell surface marker CD14 with an antibody with the appropriate fluorochrome (Table 2.3) depending on the assay. After stimulation, cells were detached from their culture plates using accutase, washed in PBS (2% FBS) and blocked with both human IgG (Merck I2511) and mouse serum (Sigma M5905). After blocking, antibodies against CD14 were added for 30 minutes, cells were then washed three times with PBS (2% FBS) and finally resuspended in PBS (2% FBS), cells were kept at 4°C throughout this process as much as possible. 30,000 events were captured, and gates were set for each cell phenotype.

Target	Cell Type	Fluorophore	Per 10^6 cells	Manufacturer (Mfr.)	Mfr. No.
CD14	Monocyte	AlexaFluor405	5µl	R&D Systems Inc.	FAB3832V
CD14	Monocyte	PE	5µl	Tonbo Biosciences	50-0149
CD14	Monocyte	AlexaFluor647	5µl	BioLegend	301812

Table 2.3 – Antibodies used for flow cytometry analysis.

2.10.2 Autophagy Assay Kit

Two autophagy assay kits were used. For HBECs, the Sigma Aldrich (MAK138) kit was used, but for monocytes the Bio-Rad (APO010A) kit was used, both kits were stored at -20°C until needed. After stimulations had finished, cells were stained with autophagosome dye according to manufacturer's protocol, blocked and antibody stained. Cells were then washed three times with PBS (2% FBS) and resuspended in PBS (2% FBS). 30,000 events were captured, gates were set based on cell type.

2.10.3 MitoSOX Assay

Intracellular mROS in HC and CF monocytes were measured and analysed by flow cytometry using Invitrogens MitoSOX assay (M36008). Monocytes were identified in the population using CD14

(Alexafluor647), MitoSOX was used to determine intracellular mROS levels in monocytes. 65.87 μl of DMSO was added to a 50 μg MitoSOX vial and diluted to a stock 1 mM in PBS. 500 μl of 1 μM MitoSOX solution (2% FBS) was added to each sample and incubated for 30 minutes, the cells were then washed twice with PBS (2% FBS), and finally resuspended in PBS (2% FBS) for analysis. 30,000 events were captured, and gates were set based on cell type.

2.10.4 Mitotracker Assays

Both Mitotracker Deep Red (MTDR) (M22426) and Mitotracker Green (MTG) (M7514) were both used at 10 nM to analyse both respiring (polarised) and non-respiring (depolarised) mitochondria respectively, both reagents were supplied by Invitrogen. Cells were incubated in 500 μl of MTDR and MTG solution (2% FBS) for 30 minutes at 37°C, washed twice with PBS (2% FBS). and finally resuspended in PBS (2% FBS) for analysis. 30,000 events were captured, gates were set based on cell type.

2.10.5 Annexin V Assay

The live/dead assay Annexin V Alexa Fluor 488 and Propidium Iodide (Thermo Fisher Scientific) was used with flow cytometry to assess whether cells were healthy, apoptotic or necrotic. Manufacturers protocol was followed. 30,000 events were captured, gates were set based on cell type.

2.11 Seahorse Metabolic Analyser

Live cell analysis of Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) using the Mito Stress Test Kit on monocytes. This assay was performed using the Seahorse XF-96 Extracellular Flux Analyser (Agilent Seahorse Bioscience). Monocytes were negatively selected using the EasySep Human Monocyte Isolation Kit produced by Stem Cell Technologies (19359) following the manufacturers separation protocol. Monocytes were suspended in Agilent Seahorse XF RPMI Medium (103576-100), the media was supplemented with glucose, pyruvate and L-glutamine.

At a concentration of $1x10^6$ cells per 1ml of media, the monocytes were seeded in quadruplicate at $1.80x10^5$ cells per well on to CellTak (Corning) coated XF-96 cell culture plates, which was prepared according to manufacturer's instructions. Stimulants supplied with the Mito Stress Kit, Oligomycin, cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and rotenone with antimycin A were used to determine basal and maximum OCR and ECAR as per the manufacturer's instructions.

2.12 Colorimetric Metabolic Assays

To analyse cellular metabolites, several Abcam colorimetric metabolic assays were utilised. ATP and L-lactate production were quantified using Abcam kits ab83355 and ab65331 respectively. Glucose consumption and succinate accumulation were analysed using Abcam kits ab136955 and ab204718 respectively. For each kit the manufacturers protocol was followed.

2.13 RNA isolation and cDNA conversion

Cells were washed in PBS and then harvested using 1 ml TRIzol™ Reagent (ThermoFisher A33251) and frozen at -80°C. RNA extraction was carried out using the Phasemaker Tubes (Thermo fisher Scientific) and following manufacturer's instructions. Upon extraction, the RNA was quantified and checked for purity using the NanoDrop spectrophotometer, taking ratio measurements at 260/280 and 260/230. RNA samples with readings above 1.75 for 260/280 ratios and 1.90 for 260/230 ratios were chosen for conversion to cDNA. The RNA was then converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following manufacturer's instructions and finally stored at -80°C until needed for RT-qPCR.

2.14 Quantitative polymerase chain reaction (q-PCR)

The genetic expression of various genes was quantified by q-PCR using the Applied Biosystems 7500 Real Time PCR System with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) (4368577) in 96 well plates. Specific reverse and forward oligonucleotide primers were designed for the genes of interest (Table 2.4) with Primer-Blast online and acquired from Integrated DNA Technologies and used at a concentration of 300 nM. The expression of mRNA was calculated relative

to the house keeping gene HPRT. Samples were added in triplicate with 20 ng of cDNA used in each reaction and all results showing only one amplification curve, to demonstrate specificity of primers.

Target	Forward Sequence (5' to 3')	Reverse Sequence (3' to 5')
LC3B	CTGTTGGTGAACGGACACAG	CTGGGAGGCATAGACCATGT
Beclin1	GGGCTCCCGAGGGATGG	CTCGTGTCCAGTTTCAGGGG
Atg5	TGACGTTGGTAACTGACAAAGTG	AATGCCATTTCAGTGGTGTG
Ulk1	GCTCTTTTGTTTCTCCGTTGGG	CGTTGCAGTACTCCATAACCA
p62	AGCAGATGAGGAAGATCGCC	CTGTAGACGGGTCCACTTCTT
BNIP3	GCCATCGGATTGGGGATCTA	CCACCCCAGGATCTAACAGC
FUNDC1	CTGGCTGGTGTGCAGGATTT	AGAAAGCCACCACCTACTGC
PINK1	GGATATGGAGCAGTCACTTACAG	GGCAGCACATCAGGGTAGTC
Parkin	CGACCCTCAACTTGGCTACT	TCTTTAATCAAGGAGTTGGGACA
Mfn1	GGAGACTTAGCATAATGGCAGA	GTGGCTATTCGATCAAGTTCCG
Drp1	GCTGCCTCAAATCGTCGTAG	TCTGCTTCCACCCCATTTTCT
NIX	CACACCAGCAGGGACCATAG	GTGGAACTCCTTGGGTGGAA
VDAC1	TCCTCCCCTTCAAATGCTGTAA	GACAACAGAAGAAGGATGAGGTT
PHB2	CAAGATGCTTGGAGAAGCACTGAG	ACCCTTGATGAGGCTGTCAC
PGAM5	GCAGGAGGAGGACAGTTACG	CGAGTGATCTTGTCGGGAGG
G6PD	CGACGACGAAGCGCAGA	TGAAGGTGTTTTCGGGCAGA
HO-1	CTGCTCAACATCCAGCTCTTTG	ATCTTGCACTTTGTTGCTGGC
NQO1	GCTGGTTTGAGCGAGTGTTC	CTGCCTTCTTACTCCGGAAGG
GCLC	ATTAGGCTGTCCTGGGTTCAC	TTTCTTGTTAAGGTACTGAAGCG
Nrf2	AACTACTCCCAGGTTGCCCA	TACAAACGGGAATGTCTGCG
KEAP1	CTCCCCAACCGACAACCAAGA	AGATAAGCAACACCACCACCT
HPRT	GGAAAGAATGTCTTGATTGTGGAAG	GGATTATACTGCCTGACCAAGGAA

Table 2.4 – Primer sequences

2.15 Statistical analysis

GraphPad Prism V7 was used for data analysis, all data are presented as the mean ± standard error of the mean (SEM), no statistical methods were used to predetermine sample size and no test was carried out to determine if samples represented a normal distribution. The statistical analysis used in this study was a two-way ANOVA multiple comparisons statistical test with Tukey correction. Comparisons were made between both HC and CF across the same stimulations, comparisons were also made between different stimulations within HC and CF respectively. Mann-Whitney non-parametric test was used to compare the median of different groups. Multiple t-test statistical analysis

with Bonferroni-Dunn correction was also used. Values were considered significant when $p \leq 0.05$ (Table 2.5). See study limitations (Section 7.6) for an explanation as to why some of these statistical tests are inappropriate.

Significance	P value
Not significant	$P > 0.05$
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$

Table 2.5 – Statistical significance key

Chapter 3.0 – Autophagy and CFTR modulators in Cystic Fibrosis HBECs

3.1 Introduction

Autophagy is a vital process in maintaining the quality of the cell membrane and intracellular organelles, thereby reducing oxidative stress, inflammation and, ultimately, preventing cell death [186]. Several papers have been published indicating that autophagy is defective in CF HBECs; however, these papers have used the IB3-1 (ΔF508/W1282X) cell line, with the C38 cell line as a WT control [79]. This is sub-optimal, as the C38 cell line is not technically a WT HBEC, as it is the CF IB3-1 cell line transfected with a WT CFTR protein, via an adeno-associated viral vector. This means that the C38 cell line has been further genetically modified, and still expresses the ΔF508 mutated CFTR protein. This will likely have unforeseen effects on cellular pathways, such as autophagy [79] and the UPR [355]. Comparing the C38 cell line to IB3-1, it was found that the number of autophagosomes was reduced in the IB3-1 cell line, formation of the late stage autophagy protein LC3B II was also reduced, and p62, which binds to ubiquitin tagged organelles and proteins targeting them for degradation, was raised; furthermore, ubiquitin was shown to be increased [79]. Accumulation of mutated CFTR into aggresomes [333] and the increase in intracellular mutated CFTR, upon proteasome inhibition [356], indicates that the lack of clearance of cytosolic mutated CFTR, and its subsequent accumulation, is not due to proteasome dysfunction but, most likely, dysfunctional autophagy.

Briefly, the mechanism by which autophagy was proposed to be defective in this comparative study was via ΔF508 CFTR forming aggregates [333], inducing an increase in production of ROS, leading to post-translational modifications of TG2; thereby, inhibiting its degradation and thus, sustaining high levels of TG2. As TG2 acts as a regulator of post translational

modifications, its overactivation was linked to the creation of cross-linkages between several autophagy proteins, and, ultimately, impairment of the autophagic mechanism [79, 331, 357].

With this in mind, it seemed pertinent to investigate the state of autophagy in several other HBEC lines, with the class II mutation ΔF508, which constitutes about 70% of mutations found in CF [8], and to establish whether autophagy is defective in the way proposed. In addition, I sought to clarify if different class mutations result in different outcomes for the state of autophagy. To date, no research has been carried out as to whether the use of CFTR modulators can improve the autophagic function of CF HBECs through partial recovery of CFTR folding and function. Furthermore, I wanted to investigate what role lysosomes might play if autophagy is defective in CF HBECs. In this chapter and proceeding chapters, the autophagic inhibitor, chloroquine, is used to freeze autophagy at the final step (Figure 3.1), allowing for characterisation of the state of autophagic flux.

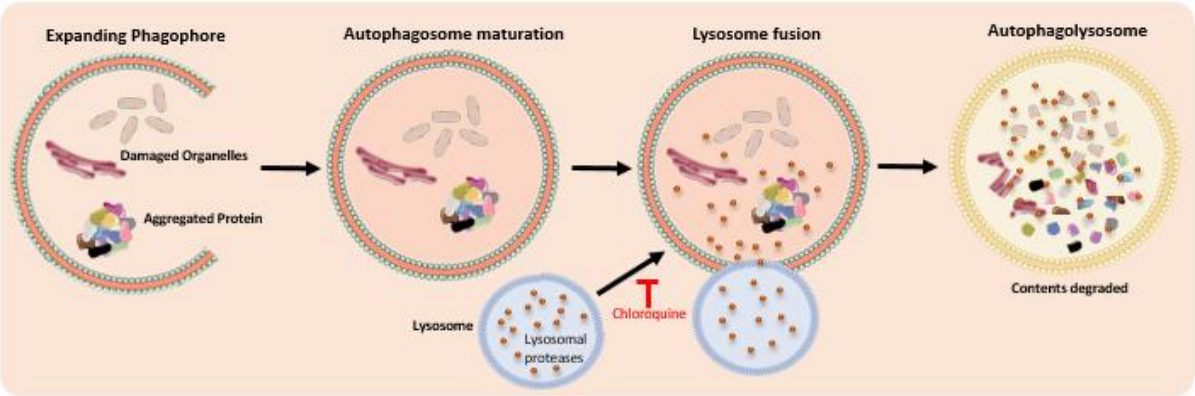


Figure 3.1 – Autophagic flux. In autophagy, an expanding double membrane, called a phagophore, encapsulates damaged organelles and aggregated protein for degradation. An autophagosome is formed once the expanding phagophore has encapsulated the cargo for degradation. A lysosome then fuses with the autophagosome, releasing the lysosomal proteases into the autophagosome, to form an autophagolysosome. The contents of the autophagolysosome are then degraded and exported back into the cell. As autophagy is a dynamic process with autophagosomes being formed and then degraded, autophagic inhibitors, such as chloroquine, are used to freeze autophagy at different stages of the pathway, providing a snapshot of the mechanism in action. Figure produced using Smart Servier Medical Art.

3.2 Results

3.2.1 Expression of autophagy genes in CF HBECs

The mRNA expression of key autophagy proteins was investigated in the Beas-2b (WT), IB3-1 (ΔF508/W1282X) and CuFi-1 (ΔF508/ΔF508) cell lines, under basal conditions. Both LC3 and Beclin1 genes were significantly upregulated in the CuFi-1 cell line (Figure 3.2), with both Atg5, Ulk1 and p62 showing no change compared to Beas-2b. The IB3-1 cell line, however, only showed a significant decrease in the expression of p62.

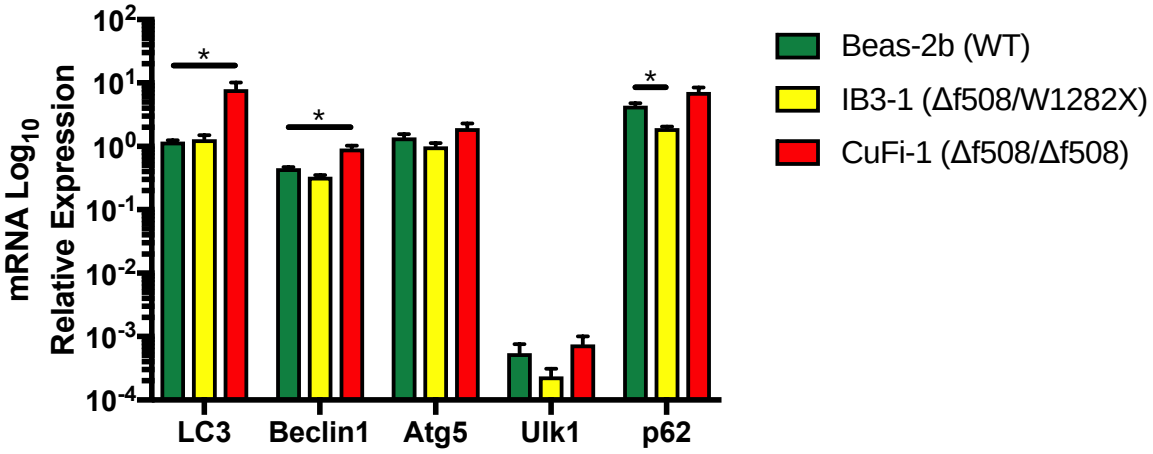


Figure 3.2 – Expression of autophagy genes in HBECs. qPCR was used to measure the expression of autophagy genes in Beas-2b, IB3-1 and CuFi-1 cell lines. mRNA expression of *LC3*, *Beclin1*, *Atg5*, *Ulk1* and *p62* were measured. All data are presented as mean ± SEM. Statistical significance was calculated using a non-parametric Mann-Whitney statistical test (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=4 for each cell line, where n is the number of experiments and passages of cell line.

3.2.2 Expression of autophagy proteins in CF HBECs

Next the protein expression of autophagy proteins was determined, using western blotting (Figure 3.3) under basal condition and with the addition of chloroquine to inhibit the degradation step of autophagy and, thereby determine the turnover rate of these proteins via autophagy. Concentrations of autophagy proteins p62, Atg5, LC3BI and II, Vps34, p150 and Beclin1 were measured. Representative western blots are shown in Figure 3.3A. The ratio of LC3BII/I was used to determine autophagic turnover, as LC3BI indicates early stage autophagy and LC3BII indicates late stage autophagy, when the autophagosome has fully formed (Figure 3.3D).

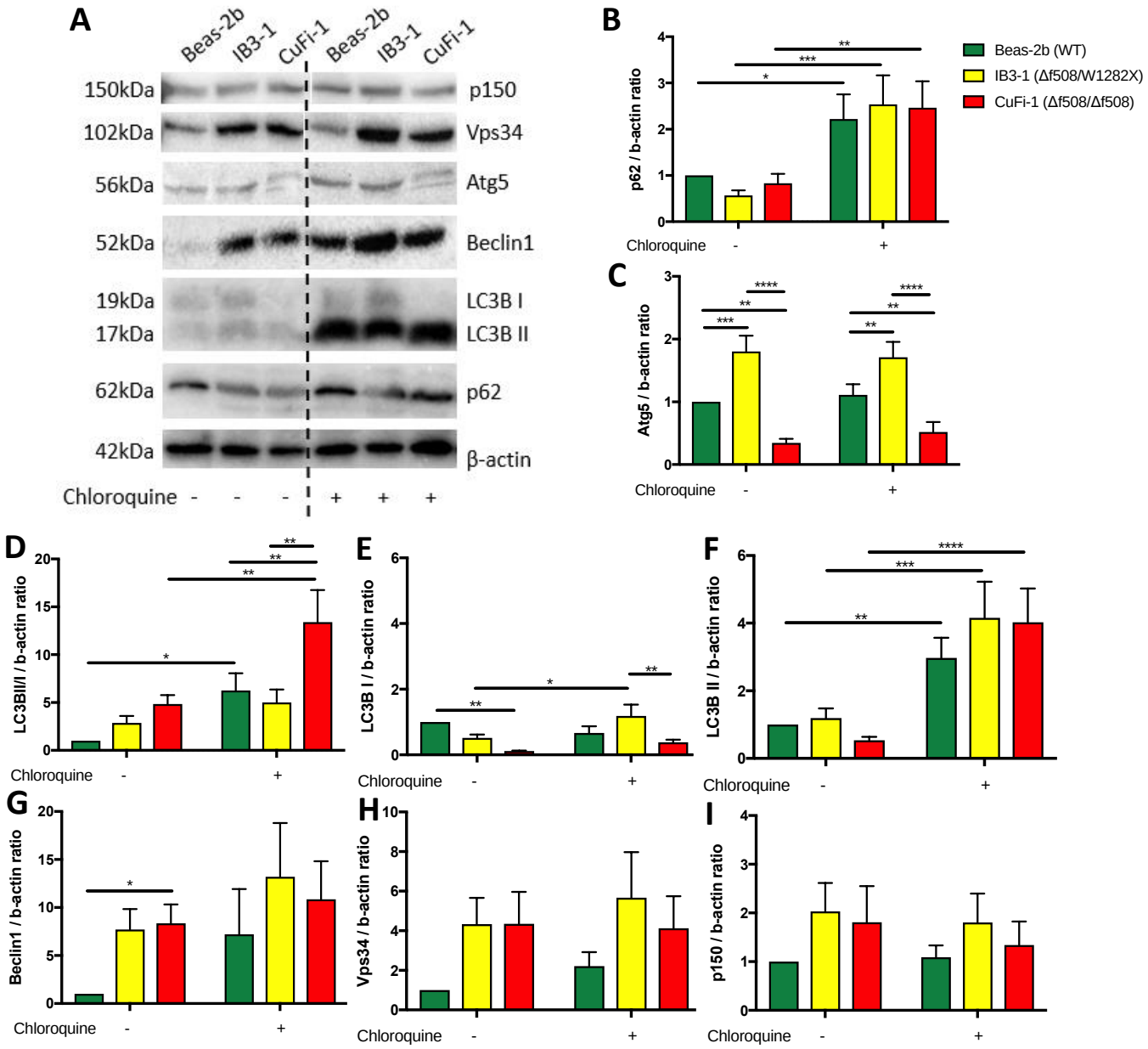


Figure 3.3 – Expression of autophagy proteins in HBECs. Western blots were used to measure the expression of autophagy proteins in Beas-2b, IB3-1 and CuFi-1 cell lines. Cells were stimulated for 3 hours with 50 μM chloroquine. Representative blots shown (A), and protein expression of p62 (B), Atg5 (C), LC3BI (E) and II (F), Beclin1 (G), VSP34 (H) and p150 (I) were normalised to β-actin and the ratios compared to Beas-2b WT. The ratio of LC3BII/I was also determined (D). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

There were no differences between any of the cell lines under basal conditions for p62, LC3BII and the LC3BII/I ratio; however, upon autophagy inhibition with chloroquine, a significant increase was shown across all cell lines (Figure 3.3B, D and F). With chloroquine, there were significant differences in the LC3BII/I ratio between the CuFi-1 cell line and both Beas-2b and IB3-1; however,

there was no significant difference between IB3-1 and Beas-2b (Figure 3.3D). Under basal conditions Atg5 appeared upregulated in the IB3-1 cell line but downregulated in CuFi-1, there was no change with chloroquine stimulation (Figure 3.3C). LC3BI was downregulated in the CuFi-1 cell line compared to Beas-2b, under basal conditions and with chloroquine (Figure 3.3E). Beclin1 was also found to be significantly upregulated in CuFi-1 compared to Beas-2b, under basal conditions, with IB3-1 showing no significant difference; however, upon chloroquine stimulation these differences disappear (Figure 3.3G). There were no significant differences, under basal conditions or with chloroquine, for Vps34 and p150 between any of the cell lines (Figure 3.3H and I).

3.2.3 Expression of autophagy proteins, with CFTR modulators in CF HBECs

The impact that the CFTR modulators, ivacaftor and tezacaftor (Symdeko), had on cell lines carrying either ΔF508/W1282X or ΔF508/ΔF508 CFTR mutations was then investigated. It was decided not to investigate Vps34 and p150 further as there were no differences established between these cell lines (Figure 3.3). Representative western blots are shown in Figure 3.4A.

With the addition of Symdeko, there was a significant increase in p62 production in the CuFi-1 cell line, compared to basal conditions, and a significant increase in p62 upon addition with chloroquine across all cell lines (Figure3.4B). p62 appeared downregulated, compared to Beas-2b and CuFi-1, upon addition of Symdeko and chloroquine (Figure 3.4B). Under basal conditions no differences were apparent between the cell lines with Beclin1, but upon addition of Symdeko, there was a significant increase between IB3-1 and Beas-2b (Figure 3.4C). Atg5 was downregulated under basal conditions in the CuFi-1 cell line, with no change upon addition of Symdeko or chloroquine (Figure 3.4D). The LC3BII/I ratio showed no difference with addition of Symdeko, with chloroquine showing an increase compared to basal, with each cell line (Figure 3.4E). There were no changes in LC3BI between the cell lines with Symdeko and chloroquine (Figure 3.4F). No change was observed in

the expression of LC3BII, on the addition of Symdeko, but there were significant differences after the addition of both Symdeko and chloroquine, between CuFi-1 and Beas-2b (Figure 3.4G).

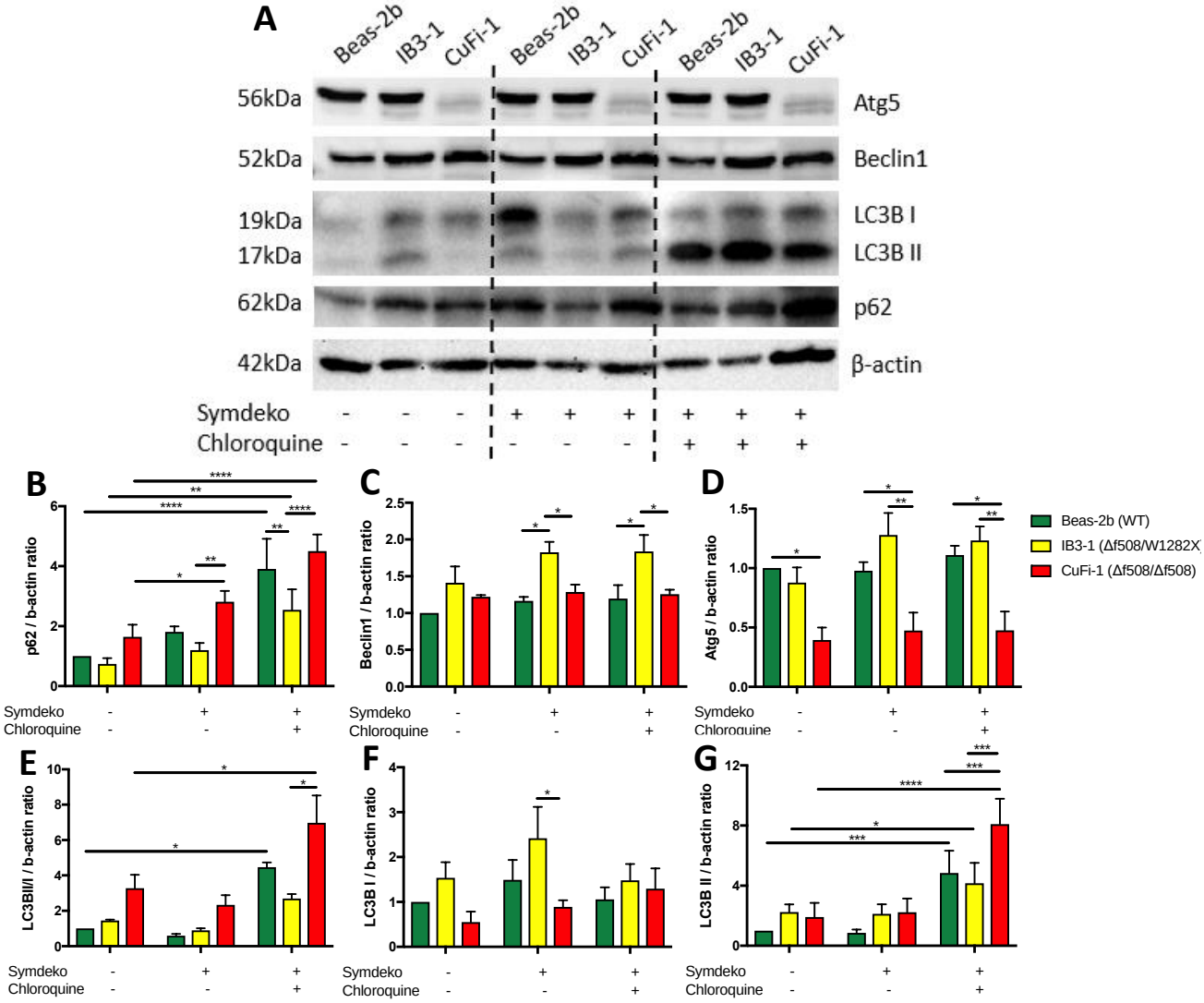


Figure 3.4 – Expression of autophagy proteins with CFTR modulators in HBECs. Western blots were used to measure the expression of autophagy proteins in Beas-2b, IB3-1 and CuFi-1 cell lines. Cells were stimulated with ivacaftor and tezacaftor (combination Symdeko) at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-stimulated at 24 hours, chloroquine was added for final 3 hours at 50 µM. Representative blots show (A). Protein expression of p62 (B), Beclin1 (C), Atg5 (D), LC3BI (F) and II (G) was normalised to β-actin and the ratios were compared to Beas-2b WT. The ratio of LC3BII/I was also determined (E). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.4 Autophagosome production in CF HBECs

Next the number of autophagosomes were quantified using flow cytometry; a representative autophagosome MFI is shown in Figure 3.5A. Under basal conditions there was no significance between IB3-1 and Beas-2b cell lines; however, the CuFi-1 cell line showed a significant increase in the number of autophagosomes (Figure 3.5B). Upon inhibition of autophagy with chloroquine, all cell lines showed a significant increase in the number of autophagosomes as well as retaining the significant difference between CuFi-1 and the other two cell lines (Figure 3.5B).

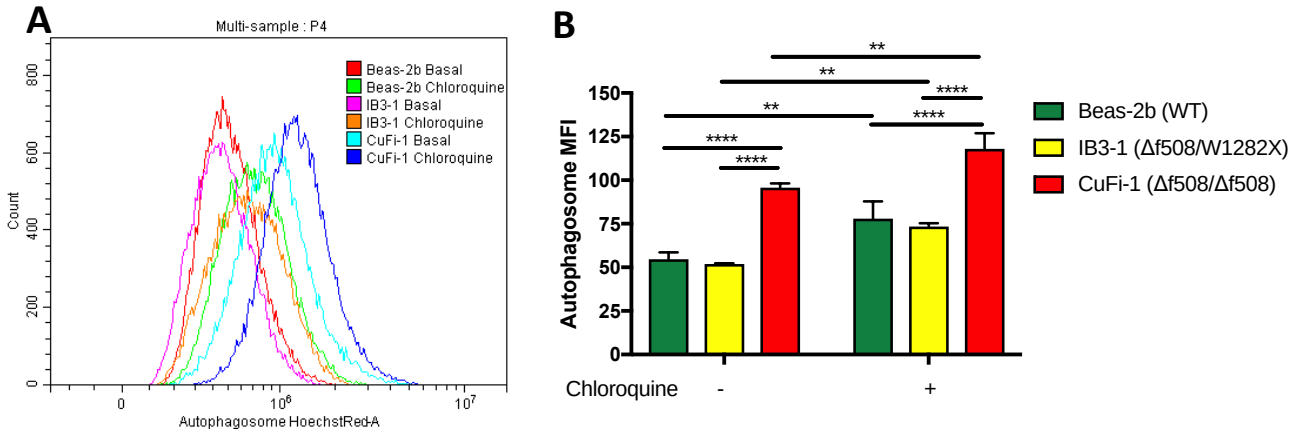


Figure 3.5 – Autophagosome production in HBECs. Flow cytometry was used to measure autophagosome production in Beas-2b, IB3-1 and CuFi-1 cell lines. Cells were stimulated for 3 hours with 50 µM chloroquine. Autophagosomes were stained with a dye supplied by Sigma. Gating strategy shown in Appendix Figure 1. Representative mean fluorescence intensity (MFI) (A) and MFI quantified (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.5 Autophagosome production with CFTR modulators in CF HBECs

Upon addition of Symdeko (representative autophagosome MFI shown in Figure 3.6A,) there was still a significant increase in autophagosome numbers between CuFi-1 and both Beas-2b and IB3-1 (Figure 3.6B). Symdeko had significantly reduced the number of autophagosomes in the CuFi-1 cell line, in comparison to basal conditions. There was no significance between IB3-1 and Beas-2b under basal conditions, with Symdeko or with Symdeko and chloroquine. Upon addition of Symdeko and chloroquine, none of the cell lines showed any increase in autophagosome number, when compared to basal conditions.

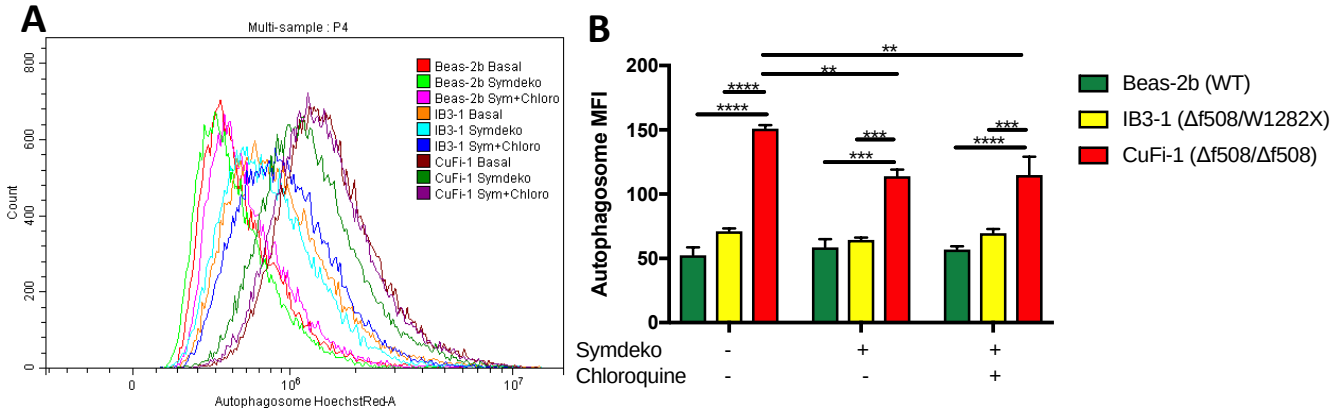


Figure 3.6 – Autophagosome production with CFTR modulators in HBECs. Flow cytometry was used to measure autophagosome production in Beas-2b, IB3-1 and CuFi-1 cell lines. Cells were stimulated with ivacaftor and tezacaftor (combination Symdeko) at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-stimulated at 24 hours, chloroquine was added for final 3 hours at 50 µM. Autophagosomes were stained with a dye supplied by Sigma. Gating strategy shown in Appendix Figure 1. Representative mean fluorescence intensity (MFI) (A) and MFI quantified (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.6 Expression of autophagy regulator with CFTR modulators

The concentration of TG2 was investigated in the Beas-2b, IB3-1 and CuFi-1 cell lines (Figure 3.7), with representative blots shown in Figure 3.7A. Western blots were quantified using densitometry; however, due to the low n number statistical analyses could not be performed.

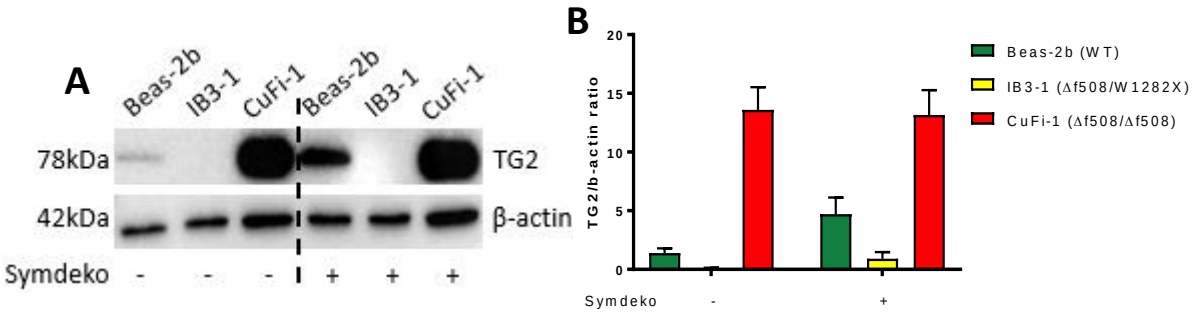


Figure 3.7 – Expression of transglutaminase 2 (TG2) in CF HBECs. Western blots were used to measure the expression of TG2 in Beas-2b, IB3-1 and CuFi-1 cell lines. Cells were stimulated with ivacaftor and tezacaftor (combination Symdeko) at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-stimulated at 24 hours. All data are presented as mean ± SEM. n=3 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.7 Lysosomal quantity and CFTR modulators in CF HBECs

At this stage, as there were no differences between Beas-2b and IB3-1, it was decided to stop investigating the IB3-1 cell line and to just focus on Beas-2b and CuFi-1. Also, as the data so far showed an upregulation in the turnover rate of autophagy in the CuFi-1 cell line, it was decided to establish whether the lysosomes were functioning normally. The number of lysosomes was quantified under basal conditions and with Symdeko, first by western blotting for the lysosomal associated membrane protein 1 (LAMP1) (Figure 3.8) and then by confocal microscopy, using Lysotracker Deep Red (LTDR) (Figure 3.9).

Representative western blots are shown in Figure 3.8A. There were no differences found between Beas-2b and CuFi-1, both under basal conditions and with Symdeko (Figure 3.8B). Confocal microscopy, representative images of Beas-2b and CuFi-1 shown in Figure 3.9A, statistical analysis was not performed due to a low n value (Figure 3.9B).

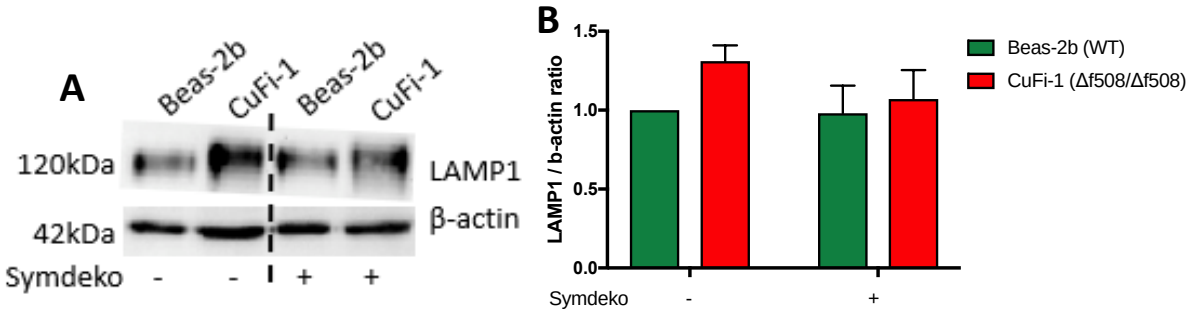


Figure 3.8 – Expression of lysosomal associated membrane protein 1 (LAMP1) with CFTR modulators in CF HBECs. Western blots were used to measure the expression of LAMP1 in Beas-2b and CuFi-1 cell lines. Cells were stimulated with ivacaftor and tezacaftor (combination Symdeko) at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-stimulated at 24 hours. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

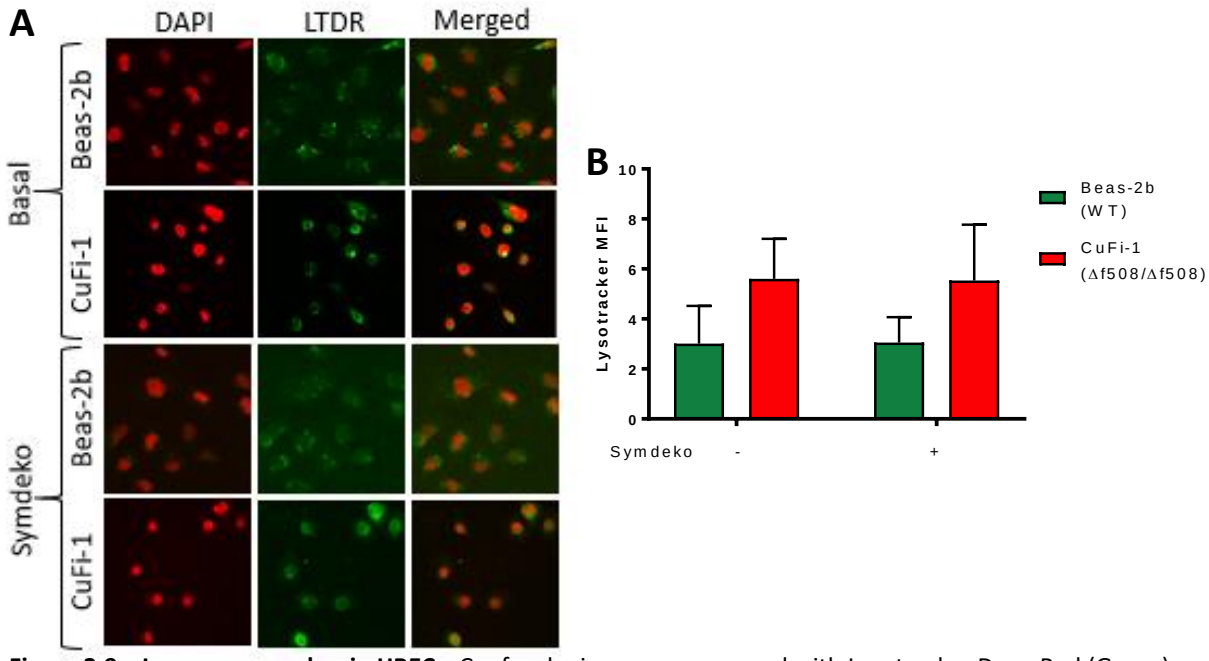


Figure 3.9 – Lysosome number in HBECs. Confocal microscopy was used with Lysotracker Deep Red (Green) to quantify the number of lysosomes within both the Beas-2b and CuFi-1 cell lines. DAPI was used to stain the nucleus (Red). All images were taken using the same parameters. Representative images shown (A) and quantification of lysosomes (B). Cells were stimulated with ivacaftor and tezacaftor (combination Symdeko) at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-stimulated at 24 hours. All data are presented as mean ± SEM. n=3 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.8 Lysosomal function and CFTR modulators in CF HBECs

To help establish lysosomal function, the activity of the lysosomal protease enzyme Cathepsin D was measured through a colorimetric assay (Figure 3.10). Under basal conditions there was no significant difference found in Cathepsin D activity between Beas-2b and CuFi-1 cell lines. Whereas, after stimulation with Symdeko, Cathepsin D activity was significantly downregulated in the CuFi-1 cell line, compared to baseline CuFi-1, but also compared to Beas-2b after Symdeko stimulation. There was no change in Cathepsin D activity in the Beas-2b cell line after Symdeko stimulation.

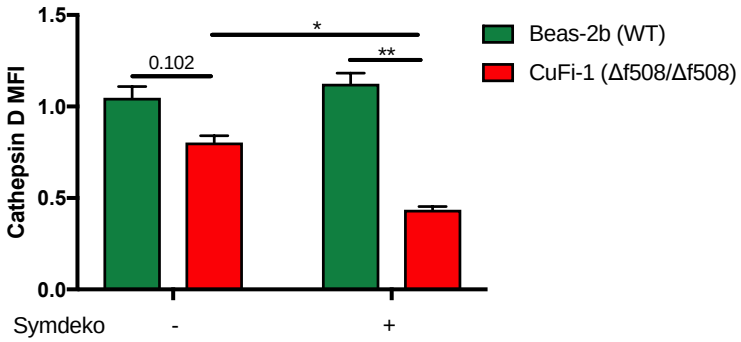


Figure 3.10 – Cathepsin D activity with CFTR modulators in CF HBECs. Colorimetric Cathepsin D assay kit was used to measure the activity of Cathepsin D in Beas-2b and CuFi-1 cell lines. Cells were stimulated with ivacaftor and tezacaftor (combination Symdeko) at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-stimulated at 24 hours. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.9 Expression of autophagy proteins in class III mutation CF HBECs

Next, the autophagic mechanism of a CF cell line (CuFi-4 (ΔF508/G551D)), with both a class II mutation (ΔF508) and a class III mutation (G551D), was investigated in comparison to Beas-2b, using western blotting; representative blots (shown in Figure 3.11A), both under basal conditions and with chloroquine added. The western blots were quantified using densitometry but due to the low n number, statistical analyses were not performed (Figure 3.11B-E).

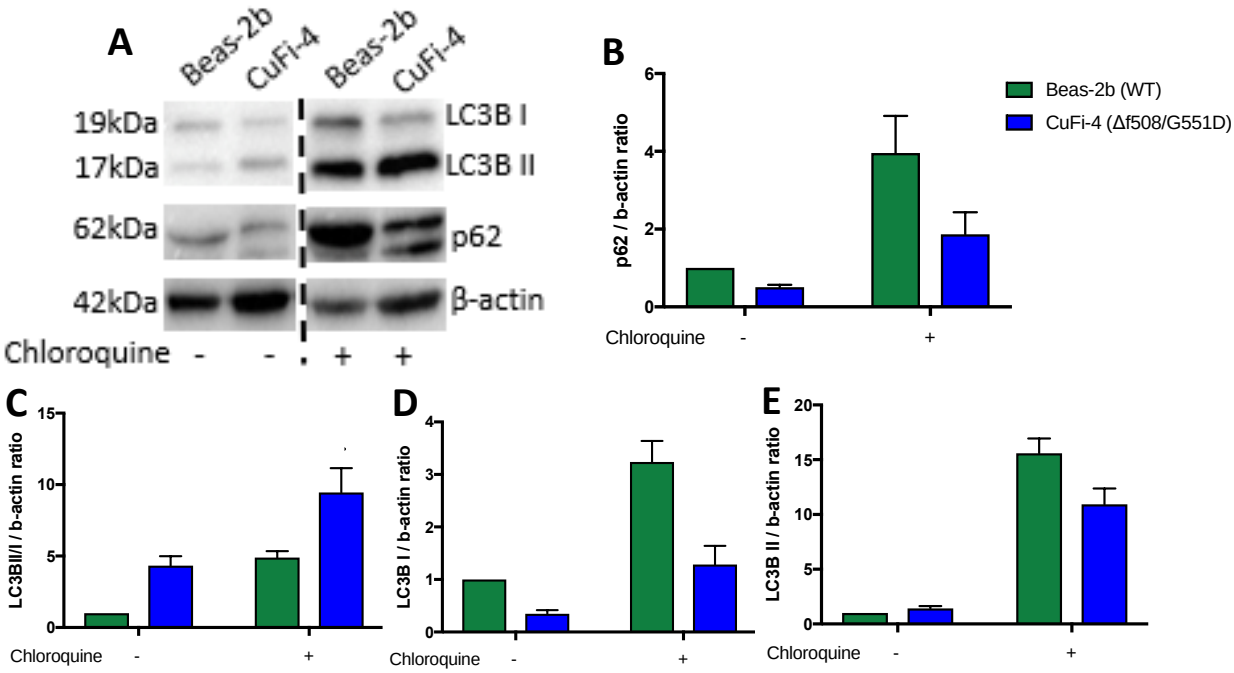


Figure 3.11 – Expression of autophagy proteins in class III mutation CF HBECs. Western blots were used to measure the expression of autophagy proteins in Beas-2b and CuFi-4 cell lines. Cells were stimulated for 3 hours with 50 µM chloroquine. Representative blots shown (A), and protein expression of p62 (B), LC3BII/I ratio (C), LC3BI (D) and II (E) were normalised to β-actin and the ratios compared to Beas-2b WT. All data are presented as mean ± SEM. n=3 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.10 Expression of autophagy proteins with ivacaftor, in class III mutation CF HBECs

The CFTR modulator, ivacaftor, which corrects the function of the CFTR protein, was used to see if just correcting CFTR function, alone, alters autophagy. Representative blots are shown in Figure 3.12A. The western blots were quantified using densitometry but due to the low n number, statistical analyses could not be carried out (Figure 3.12B-E).

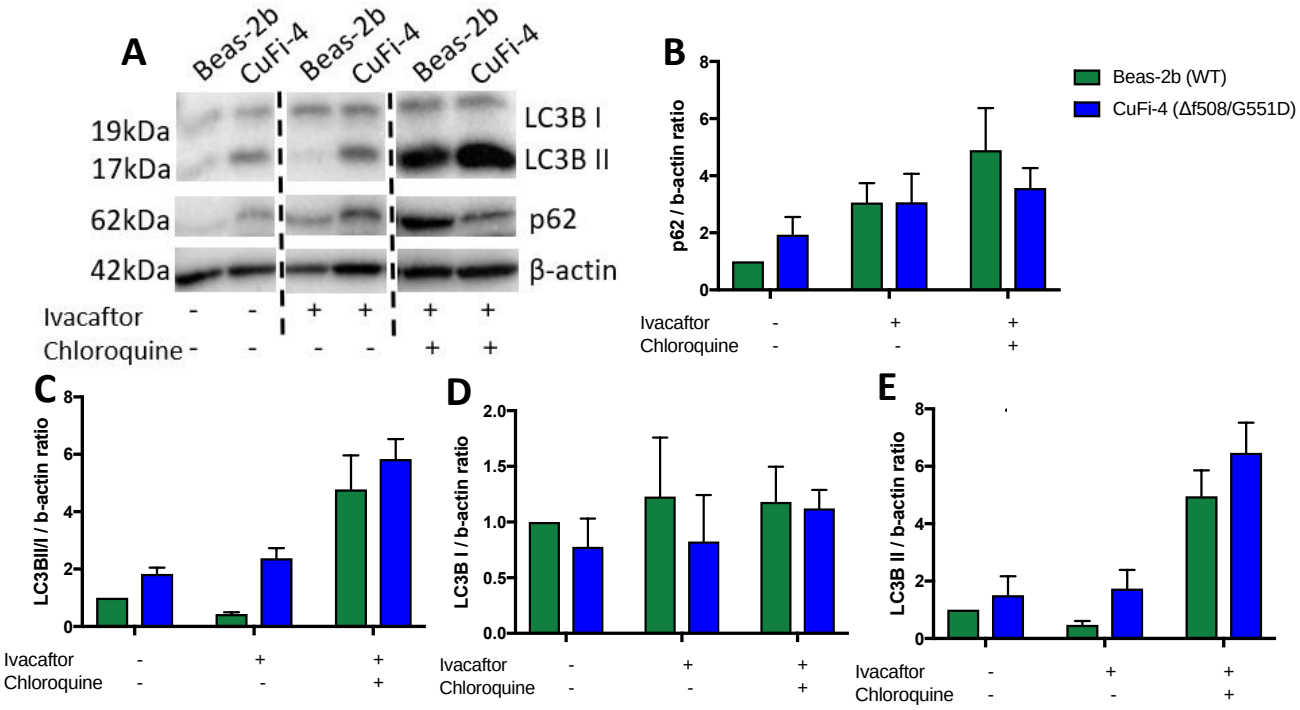


Figure 3.12 – Protein expression of autophagy with CFTR modulators in class III mutation CF HBECs. Western blots were used to measure the expression of autophagy proteins in Beas-2b and CuFi-4 cell lines. Cells were stimulated with ivacaftor at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-stimulated at 24 hours, chloroquine was added for final 3 hours at 50 µM. Representative blots show (A). Protein expression of p62 (B), LC3BII/I ratio (C), LC3BI (D) and II (E) was normalised to β-actin and the ratios were compared to Beas-2b WT. All data are presented as mean ± SEM. n=3 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.11 Autophagosome production in class III mutation CF HBECs

The number of autophagosomes produced by the CuFi-4 cell line, in comparison to the Beas-2b cell line, was then measured by flow cytometry, and representative autophagosome MFI are shown in Figure 3.13A. Due to the low number of replicates, statistical analysis was not carried out (Figure 3.13B).

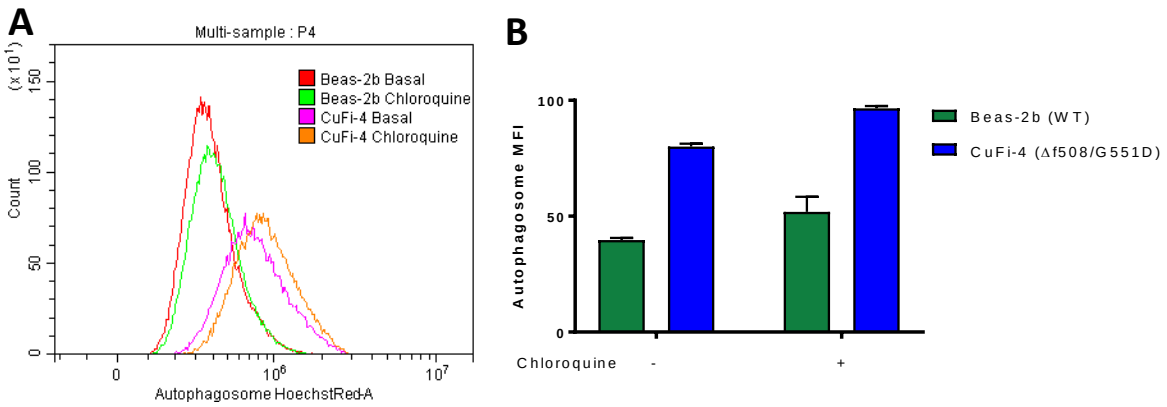


Figure 3.13 – Autophagosome production in class III mutation CF HBECs. Flow cytometry was used to measure autophagosome production in Beas-2b and CuFi-4 cell lines. Cells were stimulated for 3 hours with 50 µM chloroquine. Autophagosomes were stained with a dye supplied by Sigma. Gating strategy shown in Appendix Figure 1. Representative mean fluorescence intensity (MFI) (A) and MFI quantified (B). All data are presented as mean ± SEM. n=3 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.2.12 Autophagosome production and ivacaftor in class III mutation CF HBECs

To establish the effect of ivacaftor on the production of autophagosomes, this compound was added to both Beas-2b and CuFi-4 cell lines. Representative autophagosome MFI are shown in Figure 3.14A. Due to the low number of replicates, statistical analysis could not be performed on the data (Figure 3.14B).

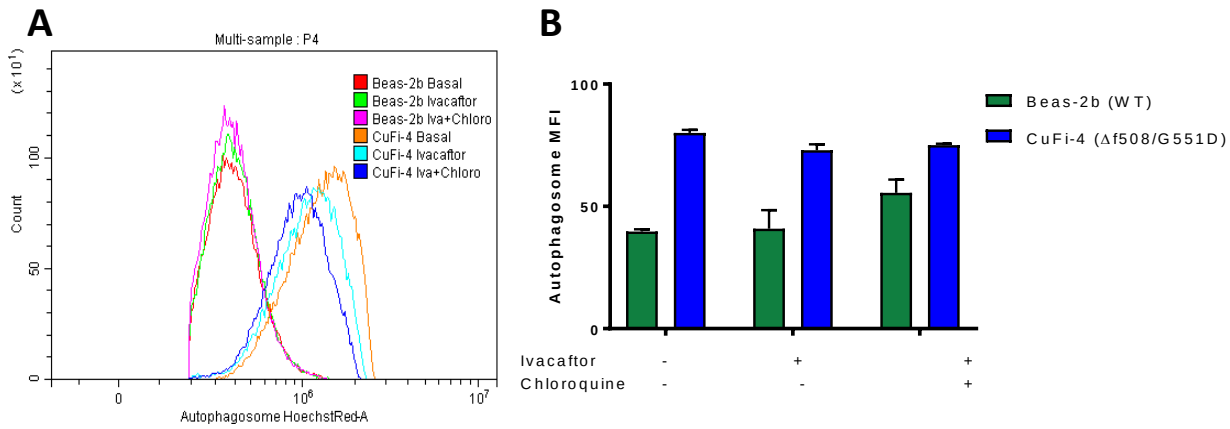


Figure 3.14 – Autophagosome production with CFTR modulators in class III mutation CF HBECs. Flow cytometry was used to measure autophagosome production in Beas-2b and CuFi-4 cell lines. Cells were stimulated with ivacaftor at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-stimulated at 24 hours, chloroquine was added for final 3 hours at 50 µM. Autophagosomes were stained with a dye supplied by Sigma. Gating strategy shown in Appendix Figure 1. Representative mean fluorescence intensity (MFI) (A) and MFI quantified (B). All data are presented as mean ± SEM. n=3 for each cell line and each stimulation, where n is the number of experiments and passages of cell line.

3.3 Discussion

3.3.1 Autophagy function in CF HBECs

As shown in Figures 3.2, 3.3 and 3.5, autophagy is clearly abnormal in the CuFi-1 but not in the IB3-1 cell line, both in comparison to the WT Beas-2b cell line' at the mRNA and protein level. This contradicts the current literature that shows autophagy is dysregulated in the IB3-1 cell line compared to the C38 cell line [79]. No statistical analyses were performed on Figure 3.11 and 3.13 due to low replicate numbers. The CuFi-1 cell line showed dysregulated autophagy, with an increased turnover ratio of LC3BII/I as well as increased production and turnover of autophagosomes (Figures 3.3 and 3.5). The turnover rate of p62, which binds to damaged organelles as well as misfolded/aggregated protein, via ubiquitin, showed no difference across the three cell lines, indicating that autophagy is removing these damaged organelles and proteins in a physiologically correct manner; furthermore, IB3-1 did not show downregulation of Beclin1, p150 or Vps34 (Figure 3.3), again contradicting the literature [79]. These data indicate that autophagy is turning over at a higher rate to that of WT in the CuFi-1 CF cell line.

Finally, as the current published model for defective autophagy in CF revolves around TG2 accumulation and overactivation, TG2 was quantified in the Beas-2b, IB3-1 and CuFi-1 cell lines (Figure 3.7). As no statistical differences could be shown due to low replicate numbers, it is not possible to make comparisons between these data and the current model of dysregulated autophagy in CF HBECs [79, 331, 357].

It is likely that the differences observed in the autophagic mechanism are due to the different genotypes found in each cell line. The fact that the CuFi-1 (ΔF508/ΔF508) cell line showed autophagy dysregulation but the IB3-1 (ΔF508/W1282X) cell line did not, implies that two protein producing mutations are required to cause dysregulated autophagy in CF, as the IB3-1 cell line has one class I mutation where no protein is produced, as well as a class II mutation. This also implies that it's the

accumulation of mutated CFTR which leads to this abnormality as opposed to the lack of CFTR function itself.

It was decided at a later stage to investigate the CuFi-4 cell line, as the initial aim was to establish whether the presence of one or two ΔF508 mutations would lead to abnormal autophagy, the G551D mutation containing cell line was added to establish whether the abnormal autophagy seen in CuFi-1 was ΔF508 mutation specific, or whether other protein producing mutations could also lead to abnormal autophagy.

However, the fact that these are different cell lines that have originated from different individuals, as well as the fact that different culturing conditions are required for some of the cell lines, makes it difficult to draw any strong conclusions. To help with this, a collaboration has been set up with Professor Fabio Martinon (University of Lausanne), to utilise CRISPR/Cas9 to create a Beas-2b cell line which is homozygous for ΔF508 as well as the creation of a CFTR KO Beas-2b cell line, as this would enable the direct analysis of whether specific CFTR mutations lead to the differences seen in autophagic control and regulation in CF.

3.3.2 Impact of CFTR modulators on autophagy in CF HBECs

As the impact of CFTR modulators on autophagy in CF hadn't been investigated before, and as there were some discrepancies between the results shown here and, in the literature, it seemed appropriate to establish what impact these modulators have on autophagy in CF HBECs.

The purpose of adding CFTR modulators to CF HBECs was to establish whether a partial correction of mutated CFTR structure and function was sufficient to correct the autophagic abnormalities seen in the CF HBECs, and whether specific correction of the function of the CFTR, in the class III mutation CF HBECs using ivacaftor, was enough to affect or alter autophagic function. As the prevailing theory behind why autophagy is dysregulated in CF HBECs is that the accumulation of mutated CFTR leads to heightened intracellular ROS and post-translational modifications of autophagy

proteins, leading to their accumulation and lack of dysfunction, I therefore hypothesised that clearing the accumulated mutated CFTR from the cell will help improve autophagy.

However, upon addition of Symdeko, there was a significant increase in p62 in the CuFi-1 cell line, with associated increased cellular stress, as well as more damaged intracellular organelles and accumulated protein (Figure 3.4), but there was also a reduction in the LC3BII/I ratio and number of autophagosomes, indicating a slower turnover rate of autophagy. This is interpreted as meaning that either Symdeko might be slowing down the rate of autophagy in the CuFi-1 cell line, as the reduction in LC3BII/I ratio implies, or that Symdeko is causing cellular stress, leading to an increase in the level of expression of p62.

The impact Symdeko had on TG2 concentration could not be analysed due to the low number of replicates (Figure 3.7).

The use of ivacaftor to correct the function of the G551D mutated CFTR protein could not be assessed due to the low number of replicates (Figure 3.12 and 3.14).

3.3.3 Lysosomal function in CF HBECs

Whether lysosomes are functioning correctly or not in CF, as well as whether the CFTR is even found in the membrane of lysosomes, are highly contentious areas of research, with various studies purporting to show either that lysosome acidification is impaired or functioning correctly [114, 358-361]; these discrepancies are largely due to disagreements over the correct methodology. The data shown above (Figure 3.8 and 3.10) show no differences in the number of lysosomes, but a reduction in the activity of the lysosomal protease enzyme, cathepsin D, indicates faulty acidification, as the enzyme was unable to function properly. The use of Symdeko to partially recover the CFTR and improve acidification of the lysosomes proved unsuccessful, which implies that the CFTR may not be present on the membrane of the lysosome.

In summary these results demonstrate that autophagy is abnormal in the CuFi-1 cell line and that comparisons between the IB3-1 and C38 cell lines should be cautiously interpreted. The data also show that abnormal autophagy requires the presence of two CFTR protein producing mutations and that the lysosomes also appear abnormal in CF. Further research into the morphological state of lysosomes and their function in CF HBECs would be highly interesting, particularly with the development of new dyes which can act as pH sensors.

Chapter 4.0 – Autophagy and CFTR modulators in CF monocytes and M1 macrophages

4.1 Introduction

The previous chapter focused on autophagy in HBEC lines and current literature surrounding that, whereas this chapter aims to clarify the state of autophagy within patients' primary cells, namely monocytes and M1 macrophages. After the publication of our groups' recent work on monocytes and M1 macrophages, in relation to the UPR [103] and hyperactivation of NLRP3 inflammasome [28], I decided to investigate the state of autophagy in these same cells, especially as there are obvious links between the UPR [362], NLRP3 inflammasome [363] and autophagy.

The current CF literature on autophagy in primary cells largely reports on mouse models and shows that the autophagy mechanism is dysregulated in BMDMs and airway epithelial cells that are homozygous for ΔF508 CFTR. The mouse models used so far have shown that ΔF508 homozygous BMDMs are unable to clear *B. cepacia* infections correctly via autophagy, and that, upon stimulation with the autophagy inducer, rapamycin, the vacuoles containing *B. cepacia* fuse with the lysosomes, aiding in the removal of infection [330]. In the same study, it was also found that *B. cepacia* downregulated several autophagy genes in BMDMs in both WT and CF mice. Depletion of the ubiquitin binding adaptor protein, p62, in BMDMs, was found to increase the clearance of *B. cepacia* infection [334]. Another report showed elevation of the microRNA cluster, Mirc1/Mirc17-92, which was shown to downregulate autophagy in mouse BMDMs. Downregulation of Mir17 and Mir20a improved the state of autophagy and also improved *B. cepacia* clearance [364]. More recently, the same group published another study, using a CF mouse model that purported to show that autophagy protein, Atg12, is highly methylated in BMDMs compared to WT, leading to weakened autophagy [337]. Airway epithelial cells in ΔF508 homozygous mice showed raised levels of p62 and decreased LC3B levels,

implying that autophagy is dysregulated; however, this particular study failed to use autophagy inhibitors to establish the turnover rate of these autophagic proteins [79].

There is limited research on autophagy in primary samples from patients with CF; Luciani *et al.* focused on nasal epithelial cell brushings, taken from patients with CF, and found both upregulated p62 and downregulated LC3BII formation, under basal conditions, indicating impaired autophagy [79]; however, again the study did not utilise autophagy inhibitors to see whether the mechanism in these human samples was turning over correctly.

Given the obvious links between the UPR, M1 macrophages and autophagy, and that, to date, there hasn't been any research on the state of autophagy in monocytes or monocyte-derived M1 macrophages from patients with CF, I decided to clarify the state of autophagy in these cell types.

4.2 Results

4.2.1 Expression of autophagy genes in CF monocytes and M1 macrophages

To establish the mRNA expression of several autophagy genes, qPCR was used for both monocytes and M1 macrophages. The expression of autophagy genes *LC3*, *Beclin1*, *Atg5*, *Ulk1* and *p62* were quantified under basal conditions (Figure 4.1A and B). There were no significant differences found between HC and CF samples for M1 macrophages; statistical analysis could not be performed on monocytes due to the low n value.

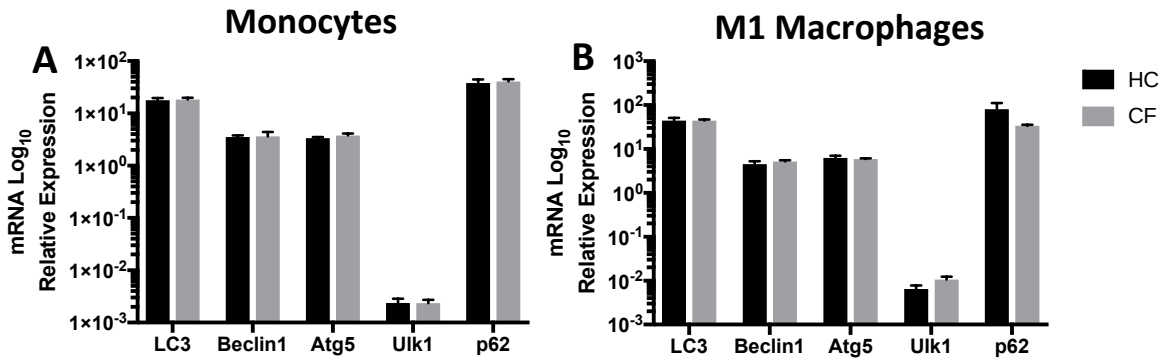


Figure 4.1 – Expression of autophagy genes in monocytes and M1 macrophages. qPCR was used to measure the expression of autophagy genes in monocytes (A) and M1 macrophages (B). mRNA expression of *LC3*, *Beclin1*, *Atg5*, *Ulk1* and *p62* were measured. All data are presented as mean ± SEM. Statistical significance was calculated using a non-parametric Mann-Whitney statistical test (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). Monocytes n=3 and M1 macrophages n=6, where n is the number of different HC or CF samples.

4.2.2 Expression of autophagy proteins in CF monocytes and M1 macrophages

The expression of autophagy proteins was then investigated in monocytes, and representative blots are shown in Figure 4.2A. Under basal conditions there was a significant increase in the quantity of p62, within CF monocytes in comparison to HC, with a significant increase in p62, in comparison to baseline, with the addition of chloroquine in HC monocytes but not in CF monocytes (Figure 4.2B). The expression of Beclin1, the LC3BII/I ratio, LC3BI and LC3BII all showed no difference between HC and CF monocytes, at baseline (Figures 4.2C-F respectively), but did show significant increases in Beclin1, LC3BII/I ratio and LC3BII, in comparison to basal conditions, when chloroquine was added (Figures 4.2C, B and F respectively). LC3BI showed no change in its expression upon stimulation with chloroquine (Figure 4.2E).

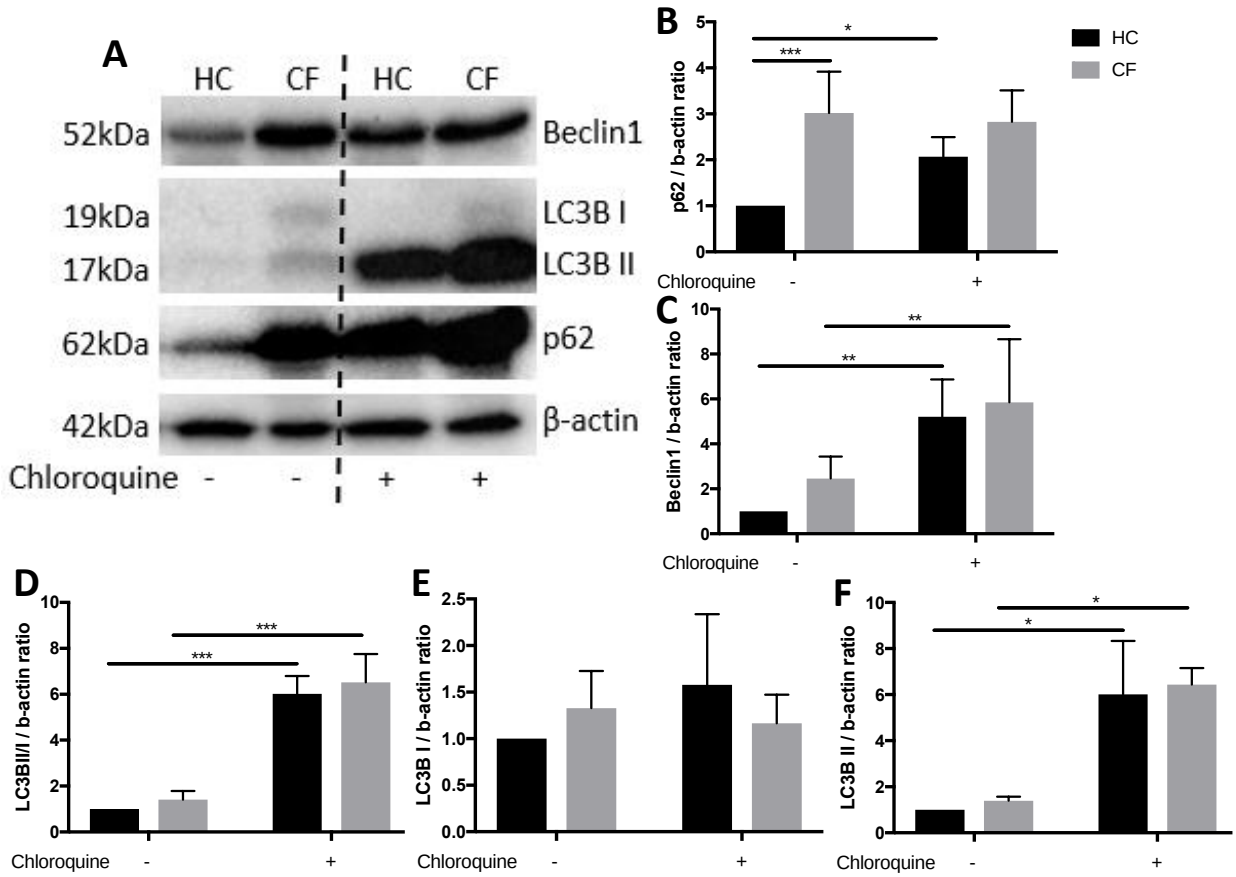


Figure 4.2 – Expression of autophagy proteins in monocytes. Western blots were used to measure the expression of autophagy proteins in both HC and CF monocytes. Cells were stimulated for 3 hours with 50 µM chloroquine. Representative blots shown (A). Protein expression of p62 (B), Beclin1 (C), LC3BII/I ratio (D), LC3BI (E) and II (F) was normalised to β-actin and the ratios were compared to HC monocytes WT. The ratio of was also determined. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=11 for p62 and LC3B, and n=5 for Beclin1, where n is the number

M1 macrophage autophagy protein expression was also measured, and representative blots shown in Figure 4.3A. Under basal conditions, the expression of p62 was significantly higher in CF M1 macrophages in comparison to HC. With the addition of chloroquine to show autophagic flux, the quantity of p62 in HC M1 macrophages significantly increased, in comparison to basal conditions, but it failed to increase in CF M1 macrophages. With chloroquine, there were still significantly higher levels of p62 in CF M1 macrophages compared to HC M1 macrophages (Figure 4.3B). At baseline, there were no significant differences between levels of LC3BII/I ratio, LC3BI and LC3BII in HC and CF M1 macrophages (Figures 4.3D-F respectively). Statistical analysis could not be performed on Beclin1 in

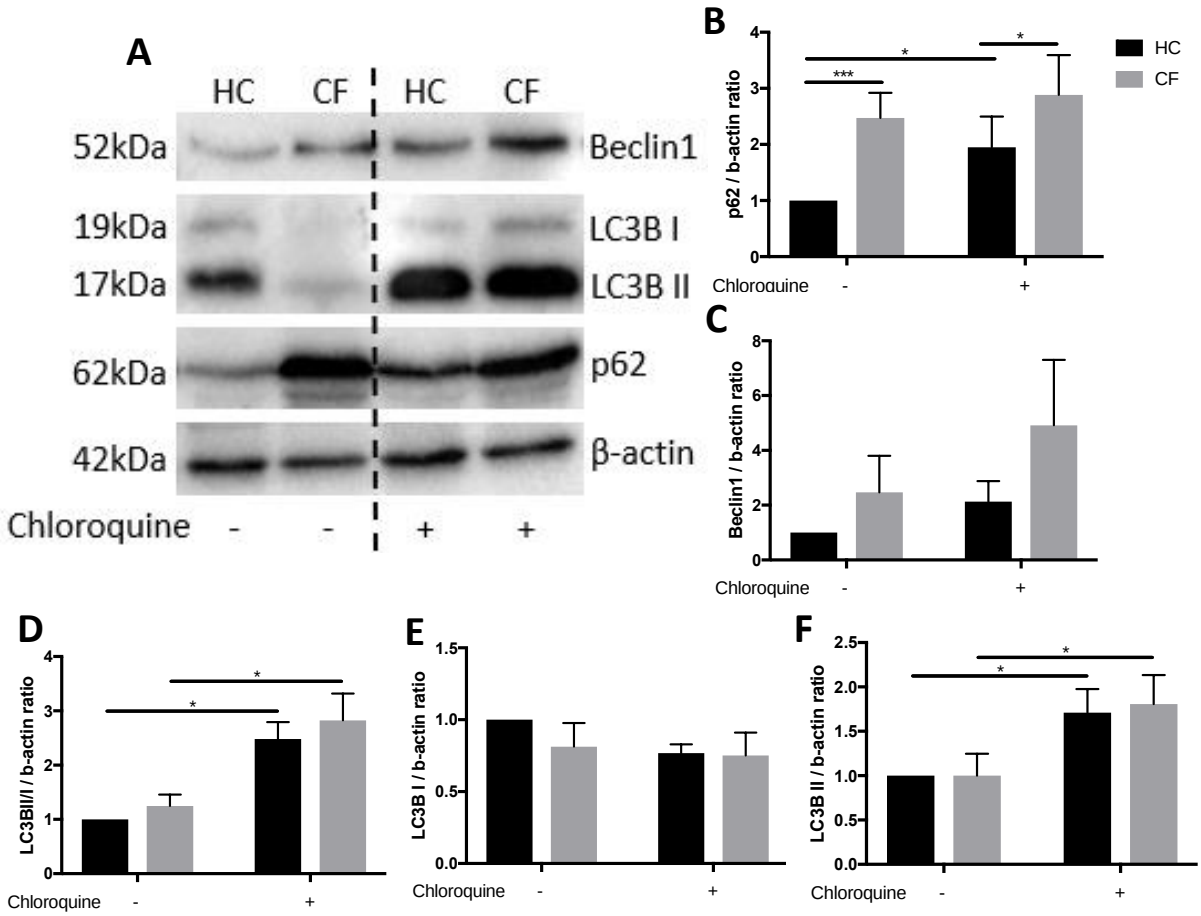


Figure 4.3 – Expression of autophagy proteins in M1 macrophages. Western blots were used to measure the expression of autophagy proteins in both HC and CF M1 macrophages. Cells were stimulated for 3 hours with 50 μM chloroquine. Representative blots shown (A). Protein expression of p62 (B), Beclin1 (C), LC3BII/I ratio (D), LC3BI (E) and II (F) was normalised to β-actin and the ratios were compared to HC monocytes. The ratio of was also determined. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=10 for p62 and LC3B, and n=3 for Beclin1, where n is the number of different HC or CF samples.

M1 macrophages due to the low number of replicates (Figure 4.3C). There was also a significant increase in both the LC3BII/I ratio and LC3BII, when chloroquine was added to both HC and CF M1 macrophages, in comparison to baseline (Figure 4.3D and F respectively). There was no change in LC3BI concentrations when both HC and CF M1 macrophages were stimulated with chloroquine (Figure 4.3E).

4.2.3 Expression of autophagy proteins with CFTR modulators in monocytes and M1 macrophages

The impact of the CFTR therapeutic, Symdeko (ivacaftor and tezacaftor), on the expression of autophagy proteins was investigated in both monocytes and M1 macrophages. Symdeko was used, instead of ivacaftor on its own, as the patients' samples used were all ΔF508 homozygous and, therefore, required a CFTR modulator that would recover CFTR's folding, and not only its function.

Representative blots for monocytes with Symdeko are shown in Figure 4.4A. Under basal conditions, p62 is significantly upregulated in CF monocytes compared to HC; upon stimulation with Symdeko there is no change between baseline and Symdeko for CF monocytes. The addition of Symdeko and chloroquine lead to a significant increase of p62 in HC monocytes but not in CF monocytes (Figure 4.4B). Both Beclin1 and LC3BI showed no difference across any stimulation or between HC and CF monocytes (Figures 4.4C and E respectively). At baseline, and following Symdeko, there were no significant differences observed between HC and CF monocytes, for both the LC3BII/I ratio and LC3BI; however, upon Symdeko and chloroquine stimulation, there was a significant increase for both HC and CF compared to baseline (Figures 4.4D and F respectively). There was also a significant difference between HC and CF monocytes with Symdeko and chloroquine (Figure 4.4F).

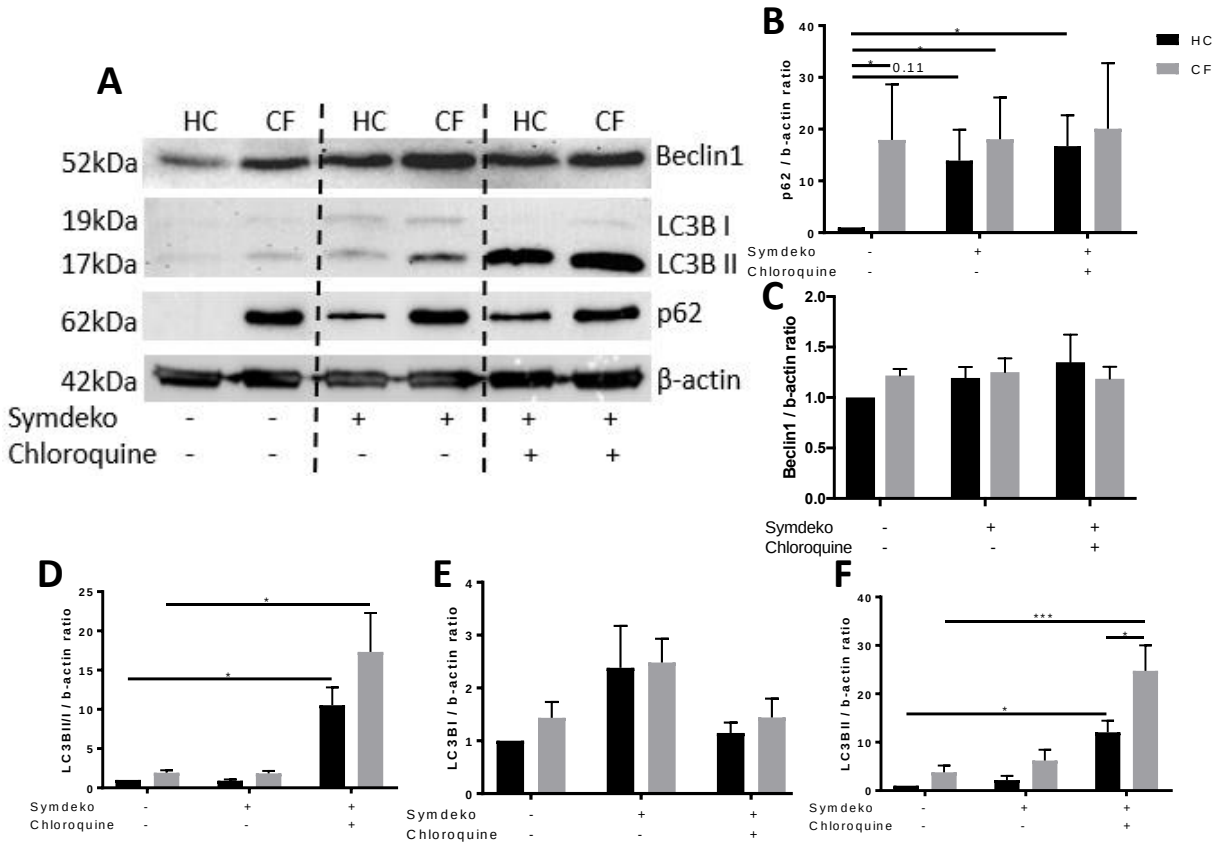


Figure 4.4 – Expression of autophagy proteins with CFTR modulators in monocytes. Western blots were used to measure the expression of autophagy proteins in HC and CF monocytes. Cells were stimulated with ivacaftor and tezacaftor (combination Symdeko) at 5 μM for 48 hours, once at time 0 hours, then with a media change and re-added at 24 hours, chloroquine was added for final 3 hours at 50 μM. Representative blots shown (A). Protein expression of p62 (B), Beclin1 (C), LC3BII/I ratio (D), LC3BI (E) and II (F) was normalised to β-actin and the ratios were compared to HC monocytes. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6, where n is the number of different HC or CF samples.

The impact of Symdeko on the expression of autophagy proteins was also investigated in M1 macrophages; representative blots, shown in Figure 4.5A, reveal that the p62 level was raised under basal conditions in CF M1 macrophages compared to HC; upon addition of Symdeko there was a significant increase in p62 in both the HC and CF M1 macrophages. When chloroquine and Symdeko were used, there is a significant increase in p62 in the HC M1 macrophages but not the CF samples in comparison to baseline (Figure 4.5B). There was no significant change found with any of the stimulations, within or between both HC and CF M1 macrophage samples, for Beclin1 (Figure 4.5C). Under basal conditions there was no significant difference between HC and CF M1 macrophages for LC3BII/I ratio, LC3BI and LC3BII expression (Figures 4.5D-F respectively). The addition of Symdeko

resulted in no change on expression for both LC3BII/I ratio and LC3BII expression (Figures 4.5D and F respectively) for both HC and CF. LC3BI showed a significant difference between CF M1 macrophages, compared to HC, following both Symdeko and Symdeko with chloroquine, under basal conditions (Figure 4.5E). Both the LC3BII/I ratio and LC3BII showed a significant increase, both upon Symdeko and chloroquine stimulation, for both HC and CF M1 macrophages (Figures 4.5D and F respectively).

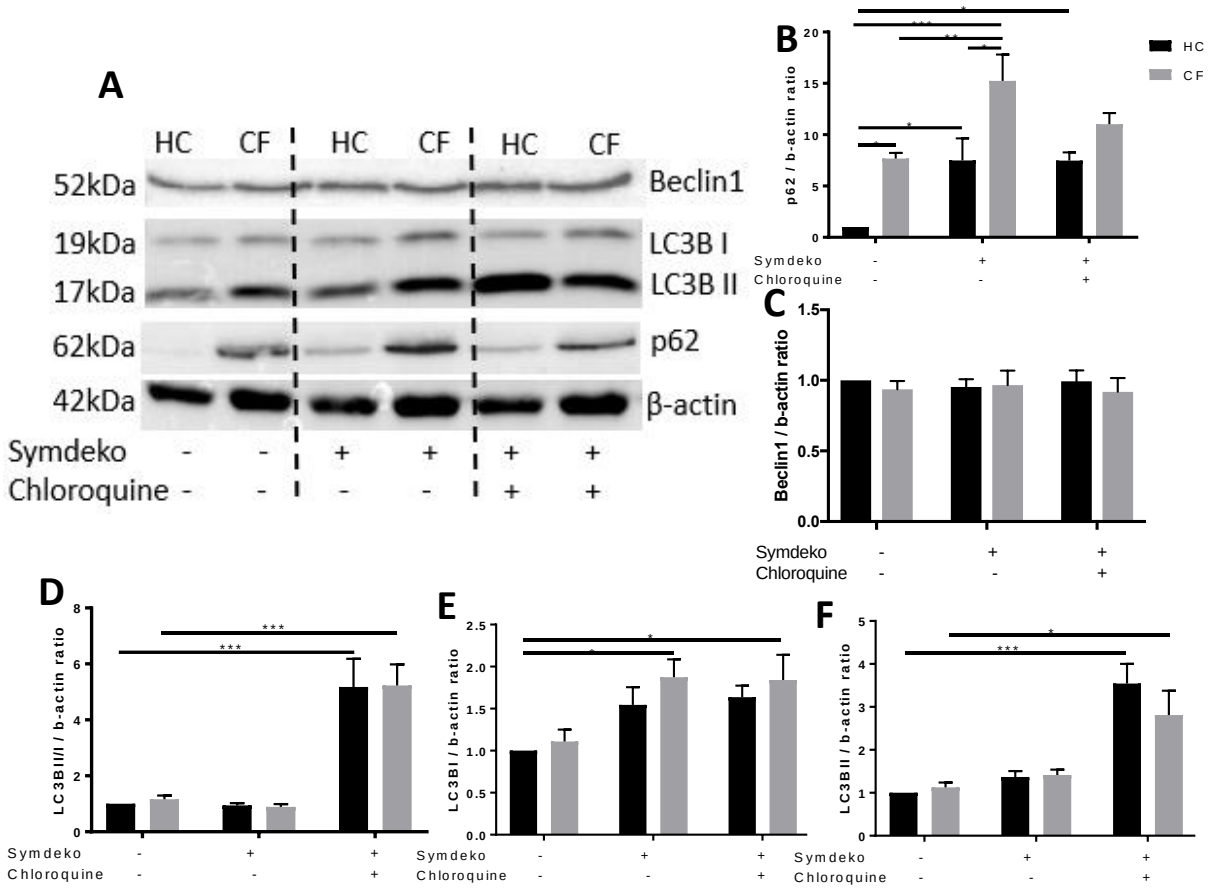


Figure 4.5 – Expression of autophagy proteins with CFTR modulators in M1 macrophages. Western blots were used to measure the expression of autophagy proteins in HC and CF M1 macrophages. Cells were stimulated with ivacaftor and tezacaftor (combination Symdeko) at 5 µM for 48 hours, once at time 0 hours, then with a media change and re-added at 24 hours, chloroquine was added for final 3 hours at 50 µM. Representative blots shown (A). Protein expression of p62 (B), Beclin1 (C), LC3BII/I ratio (D), LC3BI (E) and II (F) was normalised to β-actin and the ratios were compared to HC M1 macrophages. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8, where n is the number of different HC or CF samples.

4.2.4 Autophagosome production in CF monocytes

The production and turnover of autophagosomes in monocytes was then established, and a representative autophagosome MFI is shown in Figure 4.6A. As the state of autophagy in CF M1 macrophages seemed to be exactly the same as in CF monocytes, I decided to focus my attention purely on monocytes, so that I could develop the monocyte story to a much greater degree than if I had carried on with both monocytes and M1 macrophages. I also decided to stop using Symdeko at this point; this was because it failed to show any positive effect on the state of autophagy, in either the CF monocytes or M1 macrophages.

Under basal conditions there was no significant difference in autophagosome MFI between HC and CF monocytes; however, upon the addition of chloroquine, there was a significant increase in autophagosome MFI in both HC and CF samples compared to baseline (Figure 4.6B).

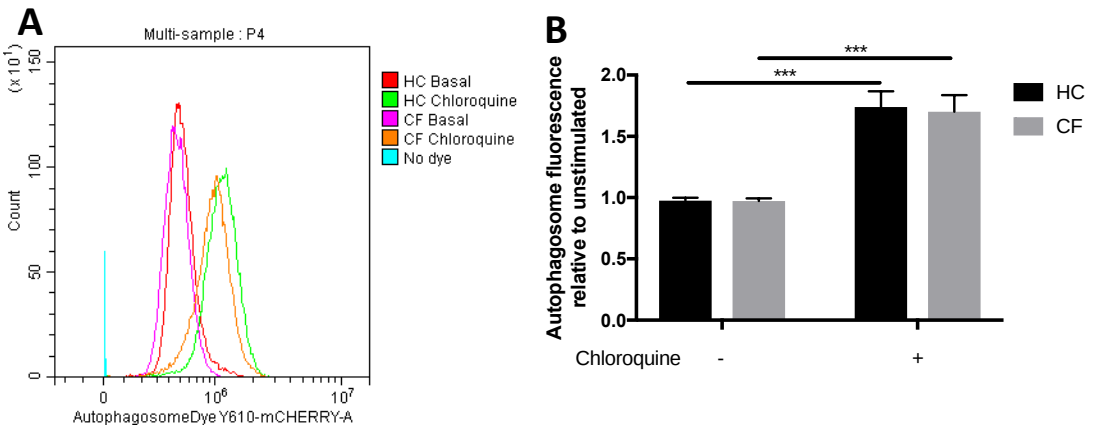


Figure 4.6 – Autophagosome production in monocytes. Flow cytometry was used to measure autophagosome production in both HC and CF monocytes. Cells were stimulated for 3 hours with 50 µM chloroquine. Autophagosomes were stained with a dye supplied by BioRad. Gating strategy shown in Appendix Figure 2. Representative mean fluorescence intensity (MFI) (A) and MFI quantified (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=4, where n is the number of different HC or CF samples.

4.2.5 Protein expression of autophagy regulator in monocytes and M1 macrophages

The expression of TG2 was quantified in both monocytes and M1 macrophages and representative blots are shown for the expression of TG2 in monocytes in Figure 4.7A. Under basal conditions there was no change in the expression of TG2 in the CF monocytes in comparison to HC samples (Figure 4.7B).

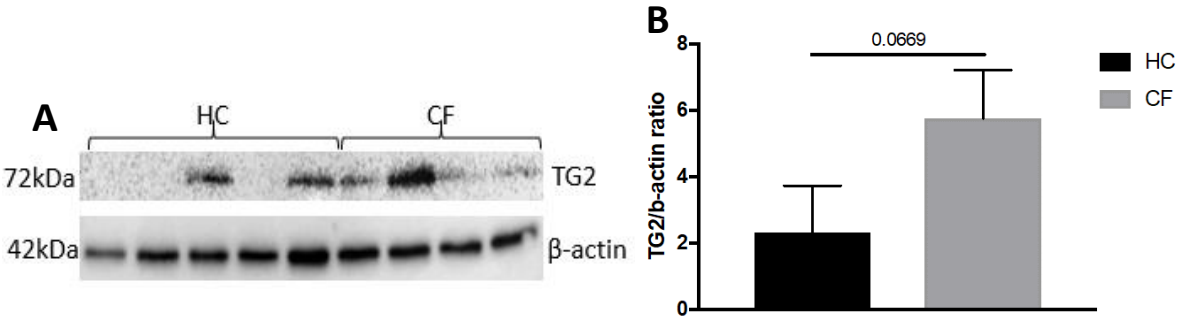


Figure 4.7 – Expression of transglutaminase 2 (TG2) in monocytes. Western blots were used to measure the expression of TG2 in both HC and CF monocytes. Representative blot shown (A), protein expression of TG2 (B) was normalised to β-actin and the ratios were compared to HC monocytes. All data are presented as mean ± SEM. Statistical significance was calculated using a non-parametric Mann-Whitney statistical test (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for HC and n=7 for CF, where n is the number of different HC or CF samples.

The expression of TG2 in M1 macrophages was also established, representative blots are shown in Figure 4.8A. There was no significance found between HC and CF M1 macrophages in the expression of TG2 under basal conditions (Figure 4.8B).

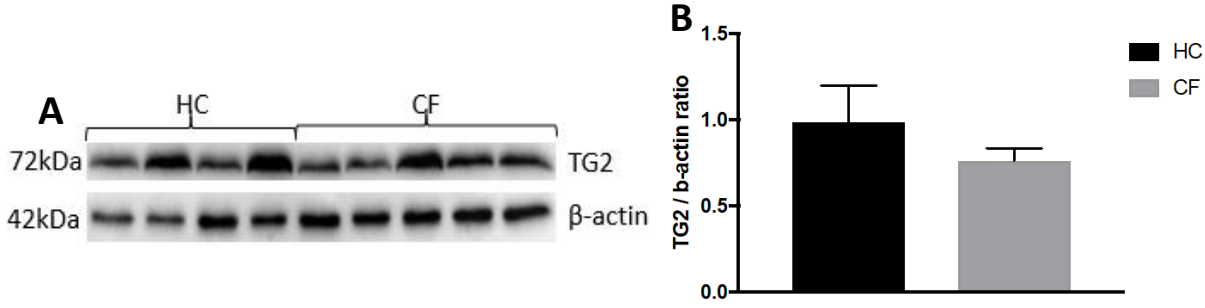


Figure 4.8 – Expression of transglutaminase 2 (TG2) in M1 macrophages. Western blots were used to measure the expression of TG2 in both HC and CF M1 macrophages. Representative blot shown (A), protein expression of TG2 (B) was normalised to β-actin and the ratios were compared to HC M1 macrophages. All data are presented as mean ± SEM. Statistical significance was calculated using a non-parametric Mann-Whitney statistical test (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for HC and n=7 for CF, where n is the number of different HC or CF samples.

4.2.6 Lysosomal quantity in CF monocytes and M1 macrophages

As no significant increases under basal conditions of p62, as well as no increase in p62 concentration upon stimulation with chloroquine, were observed in both CF monocytes and M1 macrophages, (Figures 4.2 and 4.3 respectively), it implied that p62 was not being cleared from the cell correctly. This, coupled with the observation that autophagic turnover is occurring normally with the turnover of LC3BI to LC3BII (LC3BII/I ratio), as in Figures 4.2 and Figure 4.3, and that autophagosome production and turnover is normal in CF monocytes (Figure 4.6), again indicates that the autophagic mechanism is turning over correctly and that, therefore, there might be lysosomal dysfunction instead. Therefore, I decided to establish both the quantity and function of lysosomes in both CF monocytes and M1 macrophages.

Firstly, the lysosomes were quantified in monocytes by western blotting for LAMP1; representative blots shown in Figure 4.9A; under basal conditions and with chloroquine there was no significant difference in LAMP1 expression, between HC and CF monocytes (Figure 4.9B).

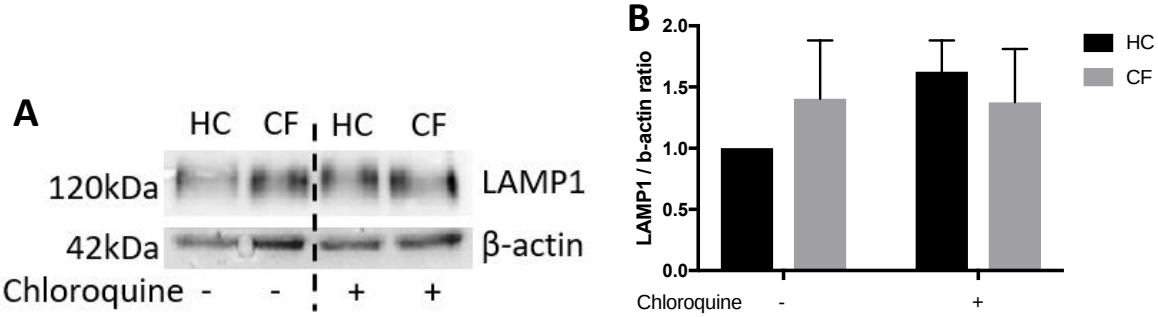


Figure 4.9 – Expression of lysosomal associated membrane protein 1 (LAMP1) in monocytes. Western blots were used to measure the expression of LAMP1 in both HC and CF monocytes. Representative blot shown (A), protein expression of LAMP1 (B) was normalised to β-actin and the ratios were compared to HC monocytes. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8, where n is the number of different HC or CF samples.

The quantity of lysosomes was also established in monocytes using LTDR with confocal microscopy, and representative images shown in Figure 4.10A. There were no significant differences found between HC and CF monocytes, under basal conditions (Figure 4.10B).

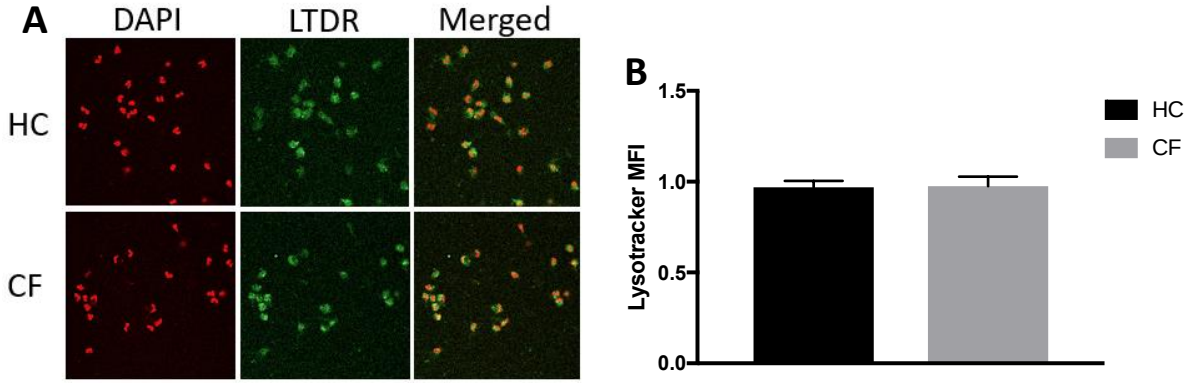


Figure 4.10 – Lysosome number in monocytes. Confocal microscopy was used with Lysotracker Deep Red (Green) to quantify the number of lysosomes within both HC and CF monocytes. DAPI was used to stain the nucleus (Red). All images were taken using the same parameters. Representative images shown (A) and quantification of lysosomes (B) was normalised to HC monocytes. All data are presented as mean ± SEM. Statistical significance was calculated using a non-parametric Mann-Whitney statistical test (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6, where n is the number of different HC or CF samples.

The expression of LAMP1 was also investigated in M1 macrophages, representative blots shown in Figure 4.11A. Under both basal conditions and with post-chloroquine there was a significant increase in the concentration of LAMP1 in CF M1 macrophages, compared to HC (figure 4.11B).

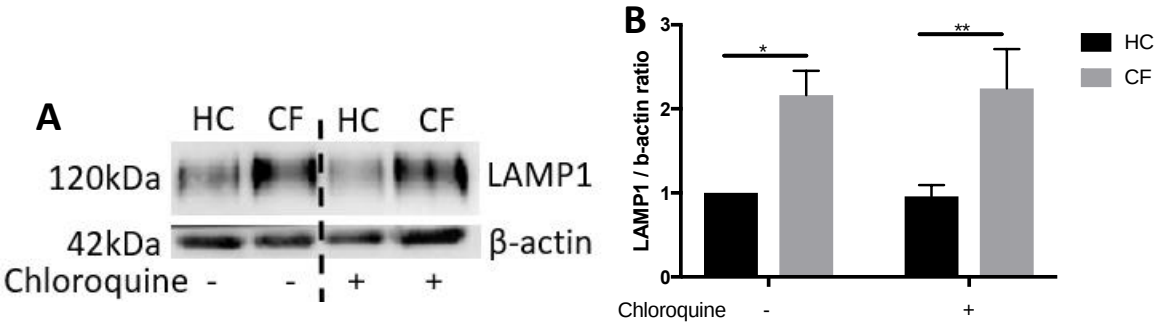


Figure 4.11 – Expression of lysosomal associated membrane protein 1 (LAMP1) in M1 macrophages. Western blots were used to measure the expression of LAMP1 in both HC and CF M1 macrophages. Representative blot shown (A), protein expression of TG2 (B) was normalised to β-actin and the ratios were compared to HC M1 macrophages. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8, where n is the number of different HC or CF samples.

The concentration of lysosomes in M1 macrophages were also established using LTDR and confocal microscopy (Figure 4.12A). There were no significant differences found between HC and M1 macrophages under basal conditions (Figure 4.12B).

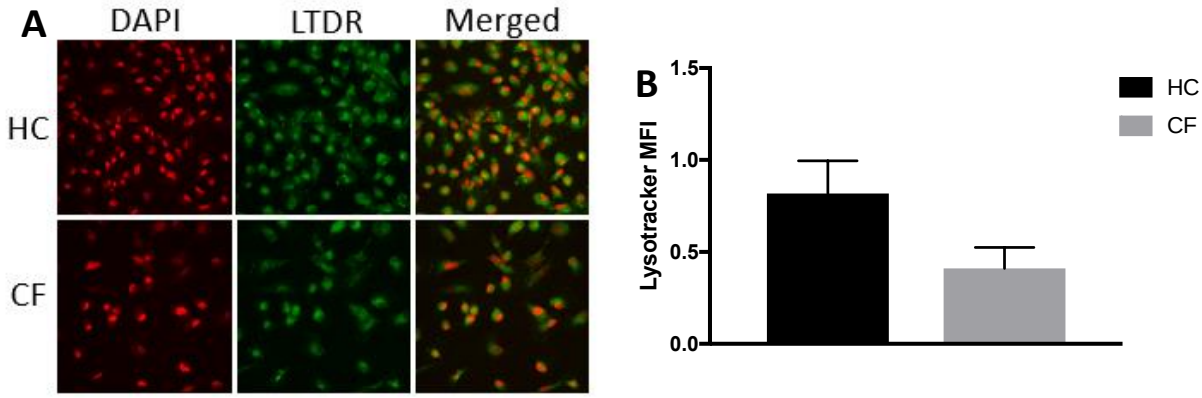


Figure 4.12 – Lysosome number in M1 macrophages. Confocal microscopy was used with Lysotracker Deep Red (Green) to quantify the number of lysosomes within both HC and CF M1 macrophages. DAPI was used to stain the nucleus (Red). All images were taken using the same parameters. Representative images shown (A) and quantification of lysosomes (B) was normalised to HC M1 macrophages. All data are presented as mean ± SEM. Statistical significance was calculated using a non-parametric Mann-Whitney statistical test (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6, where n is the number of different HC or CF samples.

4.2.7 Lysosomal function in CF monocytes and M1 macrophages

Next, lysosome function was investigated in monocytes and M1 macrophages. Activity of the lysosomal protease enzyme, Cathepsin D, was used as a readout of lysosomal function. There was no

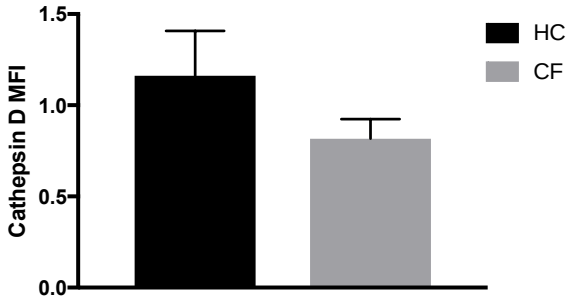


Figure 4.13 – Cathepsin D activity in monocytes. Colorimetric Cathepsin D assay kit was used to measure the activity of Cathepsin D in both HC and CF monocytes. Results were normalised to HC monocytes. All data are presented as mean ± SEM. Statistical significance was calculated using a non-parametric Mann-Whitney statistical test (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=10 for HC and n=11 for CF, where n is the number of different HC or CF samples.

significant difference in Cathepsin D activity, between HC and CF samples, for both monocytes (Figure 4.13) and M1 macrophages (Figure 4.14).

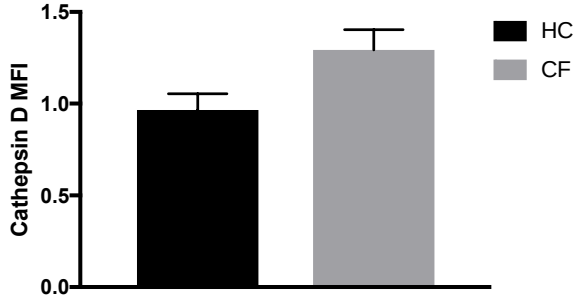


Figure 4.14 – Cathepsin D activity in M1 macrophages. Colorimetric Cathepsin D assay kit was used to measure the activity of Cathepsin D in both HC and CF M1 macrophages. Results were normalised to HC M1 macrophages. All data are presented as mean ± SEM. Statistical significance was calculated using a non-parametric Mann-Whitney statistical test (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8 for HC and n=6 for CF, where n is the number of different HC or CF samples.

4.3 Discussion

4.3.1 Autophagy function in CF monocytes and M1 macrophages

From these data, it is clear that autophagy is dysregulated in monocytes and M1 macrophages; the accumulation and lack of clearance of p62 (Figure 4.2B and 4.3B) indicates that the autophagic mechanism is unable to clear this ubiquitin binding protein from the cell. However, the turnover rate of LC3BI to LC3BII (Figures 4.2 and 4.3), as well as the turnover of autophagosomes (Figure 4.6) indicate that the mechanism is turning over properly but is unable to clear the autophagosome contents. This observation is in spite of the fact that expression of autophagy genes in CF monocytes and M1 macrophages is not dysregulated (Figure 4.1). The concentration of TG2 was investigated and found to be not significantly different between HC and CF, for both monocytes and M1 macrophages (Figure 4.7 and 4.8). This contradicts what has been published in the literature so far, as the current model for defective autophagy in CF requires TG2 to be significantly upregulated.

Between 55-80% of newly synthesised WT CFTR protein is polyubiquitinated and targeted for proteasomal degradation, due to improper folding [356]. This means that, when the CFTR is mutated, an even higher percentage of CFTR is improperly folded and targeted to the proteasome, resulting in proteasome overload [79] and cytosolic accumulation of PPARγ and mutated CFTR [79, 333, 357, 365]. p62 is a central player in regulating the formation and removal of protein aggregates [366]. Previously, raised p62 levels had been shown in CF mouse models and HBECs, and had been reported to bind to ubiquitinated ΔF508 CFTR protein, targeting it for degradation, via autophagy [79, 354]. The increased p62, found in CF monocytes and M1 macrophages, could indicate the binding of p62 to ubiquitin tagged mutated CFTR aggregates, as well as ubiquitin tagged damaged organelles, which are not being cleared from the cell in the correct manner.

The raised levels of p62, in both CF monocytes and M1 macrophages, is highly interesting. This is due to recent work showing hyperactivation of the NLRP3 inflammasome in CF [28] and previous studies showing that p62 and autophagy are both essential in regulation of NLRP3 inflammasome

activation [218, 367]. Raised levels of p62 have been linked to pathogenicity in a number of diseases, such as Gaucher disease (GD) [226], Parkinson's disease (PD) [368], systemic-onset juvenile idiopathic arthritis (SJIA) [369] and many others, all of which have distinct IL-1β signatures. p62 upregulation has also been linked to defective autophagy, and increased NF-κB activation, as well as the promotion of tumorigenesis [370].

In GD, the dysregulation of autophagy and lysosomes in macrophages has been shown to lead to an increase in p62. This increase has been linked to the over translocation of NF-κB into the nucleus, resulting in increased expression of NLRP3, as well as pro-IL-1β and pro-IL-18. p62 was also shown, in GD, to bind to the NLRP3 inflammasome component, ASC, but was unable to migrate to autophagosomes, thereby exacerbating the activation of the NLRP3 inflammasome [226]. In CF, there are inherently high levels of NF-κB [120, 371, 372] and decreased levels of the NF-κB regulators, IκBα [373] and A20 [374], which could partly be caused by the significant increase in p62.

Although NF-κB primarily upregulates the inflammatory response, it also has negative feedback loops to restrain inflammation. NF-κB can also inhibit inflammasome activation, by inducing the accumulation of p62 in LPS primed macrophages, whereby, upon activation of the NLRP3 inflammasome, p62 is recruited to damaged mitochondria. These damaged mitochondria produce NLRP3 inflammasome activation signals and are subsequently removed from the cell, via p62-mediated autophagy [367, 375]. p62 knockdown in macrophages has been shown to greatly increase the accumulation of damaged mitochondria and leads to increased IL-1β production in macrophages and neutrophils in a mouse model [367]. Therefore, the dysregulation of autophagy and subsequent accumulation of p62, may lead to damaged mitochondria, which will contribute to the excessive NLRP3 inflammasome-driven inflammatory responses seen in CF.

The elevated and sustained levels of p62 could potentially have an impact on the nuclear factor erythroid 2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein1 (KEAP1) system [376]. The transcription factor, Nrf2, and its regulator, KEAP1, constitute a major oxidative stress response

pathway, that controls the expression of important antioxidant proteins, to protect against oxidative damage to the cell. Under normal conditions, Nrf2 is bound to by KEAP1, preventing its translocation into the nucleus, by targeting it for proteasomal degradation. However, under oxidative stress p62 is upregulated and binds to KEAP1, targeting it for degradation via autophagy, thereby allowing Nrf2 to translocate into the nucleus and upregulate antioxidant proteins [377]. As p62 is greatly upregulated in CF monocytes and M1 macrophages, but oxidative stress is also prevalent in CF, it is possible that this pathway is either dysregulated or just unable to properly manage the level of oxidative stress found in CF.

As autophagy plays a central role in the differentiation of cells [378], such as monocytes differentiating in to either M1 or M2 macrophage phenotypes, dysregulation of autophagy may help to explain why the M2 macrophage population is significantly decreased in CF and why M1 macrophages present with a hyperinflammatory phenotype in CF [103].

4.3.2 Impact of CFTR modulators on autophagy in CF monocytes and M1 macrophages

CFTR modulators were used in an attempt to partially correct the structure and function of the mutated CFTR protein, reducing the accumulation of mutated CFTR within the cell, sufficiently to recover autophagy and proteostasis. The data shown above indicate that this was not the case however, as the addition of Symdeko to CF monocytes had no impact on the increased levels of p62, and significantly increased the amount of p62 in the HC monocytes, indicating that Symdeko induced cellular stress (Figure 4.4B). Stimulation of M1 macrophages with Symdeko significantly increased the quantity of p62, in both HC and CF M1 macrophages (Figure 4.5B), again showing that Symdeko may be stressing the cells, possibly increasing protein and organelle ubiquitination. In both monocytes and M1 macrophages, Symdeko had no impact on the turnover of LC3BI to LC3BII (Figure 4.4D-F and 4.5D-F respectively), indicating that it didn't alter autophagic flux. These results could potentially be due to Symdeko being added at too high a concentration; however, previous work has used the same concentration (5 μM) of these modulators (ivacaftor and tezacaftor) [63, 379]. I also wanted to assess

the effect of the highly effective CFTR modulator combination elexacaftor, tezacaftor and ivacaftor but was unable to gain access to these medications through Vertex Pharmaceuticals.

4.3.3 Lysosomal function in CF monocytes and M1 macrophages

As autophagosome production and turnover appeared normal, but p62 was still accumulating within the cells, I decided to investigate the state of lysosomes within CF monocytes and M1 macrophages. There was no difference in the quantity or function of lysosomes in either CF monocytes (Figures 4.9, 4.10 and 4.13) or M1 macrophages (4.12 and 4.13) compared to HC, apart from the concentration of LAMP1 in CF M1 macrophages which was significantly raised (Figure 4.11).

CFTR has been shown to associate with LAMP1 [360, 380] and has been found to be essential to the acidification of phagosomes in neutrophils [380]. So, it is possible that the increased expression of LAMP1 in the M1 macrophages is to compensate for the lack of functioning CFTR protein available to bind to and facilitate the acidification of phagosomes. CF macrophages have been found to be unable to phagocytose and kill pathogens, for example *S. aureus*, indicating the presence of autophagic and lysosomal dysfunction [381]. The reason why an increase in LAMP1 in CF monocytes is not found could be due to macrophages playing a much greater role in phagocytosis.

Previously, LAMP1 expression has been shown to be downregulated in CF HBECs and non-responsive to LPS stimulation [382], whereas, in COPD patients' primary lung epithelium samples, LAMP1 has been found to be raised and is associated with decreased lung function [383]. This difference may be due to variations in study methodology, where the CF study focused on cell lines and the COPD study was performed on primary samples.

Chapter 5.0 – Oxidative stress and mitochondrial function in CF monocytes

5.1 Introduction

Oxidative stress is a key driver of inflammation in several diseases, such as cardiovascular disease [384], neurodegenerative disease [385], COPD [386] and CF [387]. Oxidative stress has also been found to reduce CFTR mRNA stability and protein expression [388]. There are several mechanisms that cells use to manage oxidative stress, with one of the main mechanisms being the Nrf2 pathway [389].

The Nrf2 pathway represents a key anti-inflammatory mechanism, whereby anti-oxidant proteins are upregulated, NF-κB is downregulated and NLRP3 inflammasome activity is inhibited [389]. Under basal conditions, Nrf2 is bound to by KEAP1, ubiquitinating Nrf2 and targeting it for degradation via the proteasome [390-392]. In contrast, upon oxidative stress, KEAP1 undergoes modification of specific cysteine residues, leading to its dissociation away from Nrf2 and its subsequent translocation into the nucleus, to upregulate the expression of anti-oxidant genes [393]. This pathway is referred to as the canonical Nrf2 mechanism; however, the non-canonical Nrf2 pathway involves the interaction of KEAP1 and p62. Upon oxidative stress, p62 is upregulated and binds to KEAP1, targeting KEAP1 for its degradation via autophagy, which prevents proteasomal degradation of Nrf2, allowing its nuclear translocation (Figure 5.1) [394-397].

One of the main targets of Nrf2 activation is increased expression of an anti-oxidant enzyme, heme oxygenase (HO)-1. HO-1 is the rate limiting enzyme for degradation of biliverdin into bilirubin, and heme into carbon monoxide (CO) and iron. Free heme acts as a DAMP, causing oxidative damage and inflammation, via activation of innate immune cells, whereas CO and bilirubin have anti-inflammatory properties. CO has been shown to inhibit NF-κB activation in monocytes, after LPS activation [398], and bilirubin has been reported to have potent anti-oxidant properties [399]. Furthermore, HO-1 downregulates inflammatory cytokine expression [400] and upregulates anti-

inflammatory cytokine expression [400, 401]; therefore, the activation of HO-1 is an important homeostatic mechanism.

Other Nrf2 target genes include NADPH dehydrogenase quinone (NQO)1, which reduces ROS generating quinones to hydroquinones, making the removal of quinones an important anti-oxidant mechanism [402]. Glutamate cysteine ligase catalytic (GCLC) is another Nrf2 target gene and is the rate limiting step enzyme for production of the anti-oxidant, glutathione (GSH) [403]. Another Nrf2 target gene, glucose-6-phosphate dehydrogenase (G6PD), is essential for the production of NADPH and maintaining cellular redox homeostasis; a deficiency in G6PD has been shown to lead to defective autophagy, excessive inflammation, insulin resistance and infection [404].

The Nrf2 pathway is also a key regulator of cytokine secretion, as it prevents LPS-induced upregulation of IL-6 and IL-1β; moreover, in a mouse model, KO of Nrf2 results in increased IL-6 and IL-1β production [405, 406]. The Nrf2 target genes, HO-1, NQO-1 and GCLC have been shown to inhibit production of IL-6, TNF, monocyte chemoattractant protein (MCP)-1, in a mouse model [407]. Nrf2 has also been reported to regulate activation of the NLRP3 inflammasome, via upregulation of NQO1 which inhibits the accumulation of ROS and subsequent NLRP3 priming [408]. However, a study in 2014, demonstrated that, as well as being involved in inhibition of NLRP3 inflammasome activation, the Nrf2 pathway is also required for activation of both the NLRP3 and AIM2 inflammasomes but not the NLRC4 inflammasome [409].

Previously, Nrf2 expression and function has been shown to be downregulated in CF airway epithelial cells, with raised cytokine secretion and H_2O_2 production [410, 411]. However, to date, little research has been devoted to the study of whether the Nrf2 pathway is functioning normally, in primary monocytes and monocyte derived M1 macrophages from CF patients. In a mouse model, the knockdown of Nrf2 has been reported to lead to a reduction in CFTR expression [412]. Inhibition of the function of CFTR with $CFTR_{inhib}$-172 has also been found to decrease activity of Nrf2 in epithelial cells [410]. Use of tezacaftor or lumacaftor to correct the misfolding of ΔF508 mutated CFTR has been

reported to recover Nrf2 activity, leading to increased expression of *NQO1* and *GCLC* [413]. A recent study demonstrated binding of the Nrf2-KEAP1 complex to the mitochondrial membrane protein serine/threonine-protein phosphatase (PGAM)5, and this interaction was required for trafficking of mitochondria to areas of high metabolic activity [414, 415]. PGAM5 has also been shown to be a central player in driving mitochondrial dysfunction in lung fibrosis in mice [416].

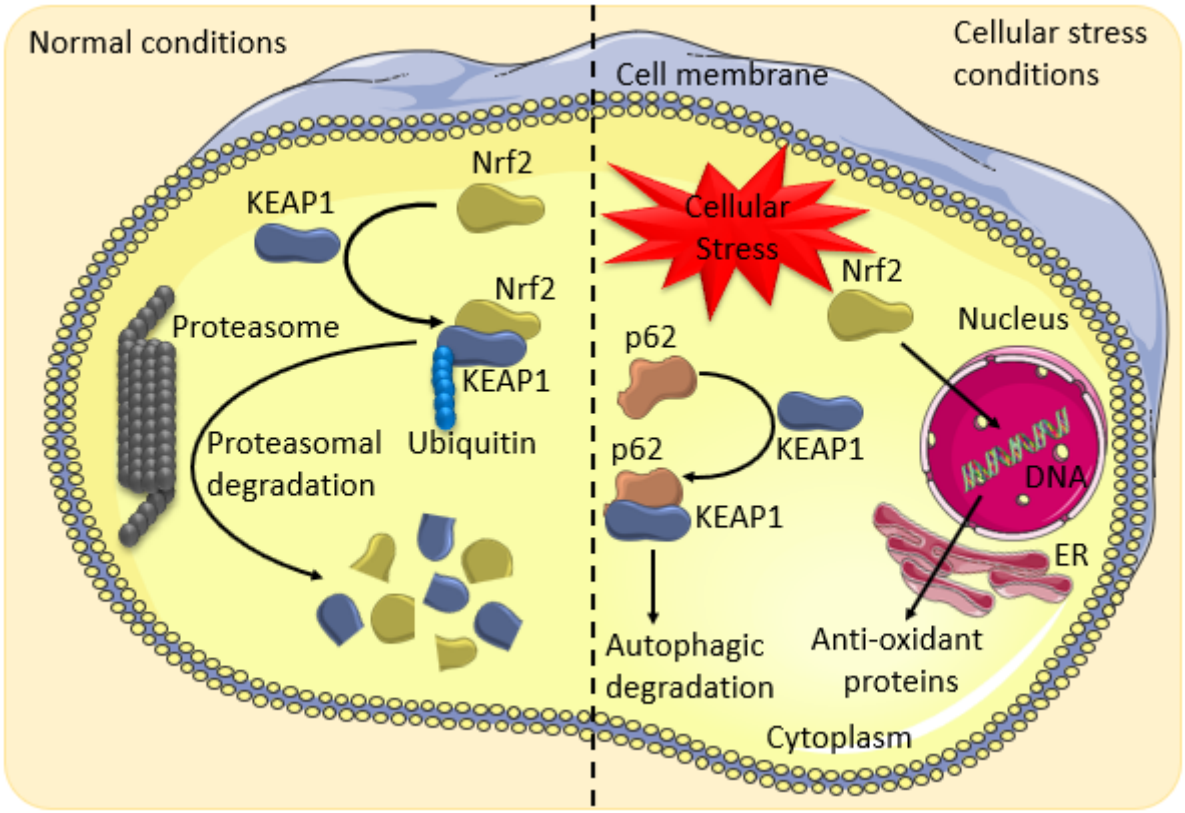


Figure 5.1 – Non-canonical Nrf2-KEAP1 oxidative stress pathway. The non-canonical nuclear factor erythroid 2-related factor (Nrf)2 pathway represents a key anti-oxidative stress pathway. Under normal conditions Kelch-like ECH-associated protein (KEAP)1 binds to and ubiquitinates Nrf2, targeting it for proteasomal degradation. Upon cellular stress, p62 is upregulated and binds to KEAP1, preventing it from binding to Nrf2, targeting KEAP1 for autophagic degradation. Nrf2 translocates into the nucleus to upregulate the expression of several anti-oxidant proteins, such as, heme oxygenase (HO)-1, NADPH dehydrogenase quinone (NQO)1, glutamate cysteine ligase catalytic (GCLC), phosphoglycerate mutase family member (PGAM)5 and glucose-6-phosphate dehydrogenase (G6PD). Figure produced using Smart Servier Medical Art.

Several clinical trials investigating the clinical benefit of anti-oxidants GSH, β-carotene, selenium, as well as vitamin E and C, have delivered conflicting results as to their efficacy, with a Cochrane review concluding that more research is needed [417, 418]. A recent clinical trial sought to

elucidate whether GSH levels are low in BALF in young children with CF, as well as investigating the extent of the role oxidation plays in GSH deficiency and what effect respiratory infections might have on GSH levels. The authors found a strong correlation between bronchiectasis and MPO, in BALF and glutathionylation of airway proteins [419].

One of the key drivers of oxidative stress and inflammation is damaged/depolarised mitochondria, evidenced by the fact that several autoinflammatory diseases present with damaged mitochondria and altered metabolism [158, 159, 164, 170, 171, 180]. Defective autophagy also plays an important role in the pathogenicity of these diseases, as there is a failure to clear damaged/depolarised mitochondria, exacerbating the inflammatory phenotype further [160, 161, 163, 170, 171, 231].

Dysregulation of autophagy in CF patients' primary cells in the previous chapter led to my hypothesis that there may be some associated mitochondrial abnormalities and heightened oxidative stress, due to lack of clearance of damaged/depolarised mitochondria. Mitochondrial abnormalities would be likely to add to the exacerbated inflammation already seen in CF, with excessive activation of the NLRP3 inflammasome [28], as well as an altered metabolic state [103]. Furthermore, mitochondria have been found to be central players in the development of several lung diseases [420]. Damaged mitochondria not only release NLRP3 inflammasome activating signals, such as mROS and mtDNA (Figure 5.2), but also act as a platform for NLRP3 inflammasome formation [421, 422].

Binding of ATP to P2RX7 receptor, leading to K^+ efflux, has been shown to increase intracellular Ca^{2+}, alter mitochondrial metabolism and may damage mitochondria [320-322], as well as the addition of ATP leading to production of mROS [423]. Mitochondria have also been revealed to activate the NLRP3 inflammasome, independently of K^+ efflux [424].

NLRP3 inflammasome activation in human macrophages, with LPS and ATP (Figure 5.2), induced an association between p62 and a component of the NLRP3 inflammasome, called ASC, targeting this component for degradation via autophagy [218]. Whereas dysregulated autophagy

leads to an accumulation of p62, and possible build-up of damaged mitochondria, the knockdown of p62 in macrophages has been shown to lead to an accumulation of damaged mitochondria and enhanced IL-1β production [367], highlighting the delicate balance of p62 expression in mitochondrial function.

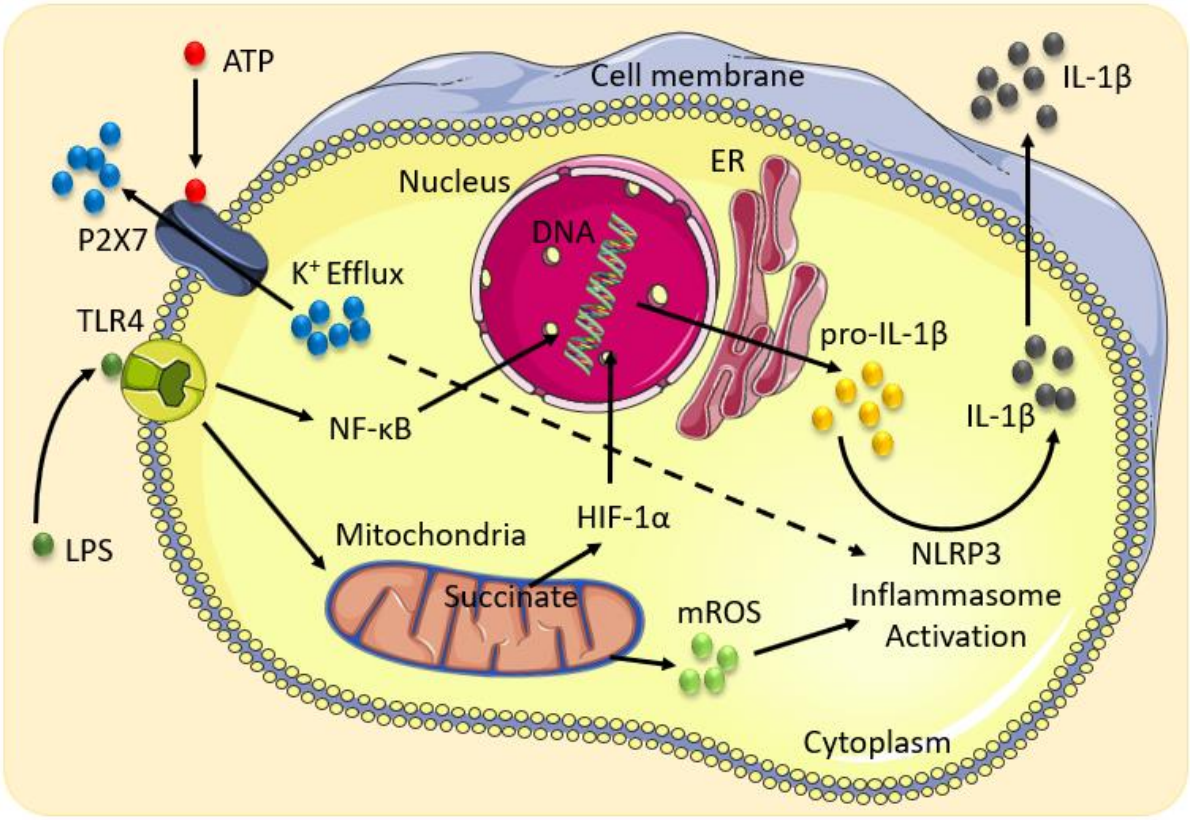


Figure 5.2 – NLRP3 inflammasome activation. Activation of the NLRP3 inflammasome is triggered in response to two signals, pathogen associated molecular patterns (PAMPs), such as LPS, and danger associated molecular patterns (DAMPs), such as ATP. LPS acts as a PAMP for the NLRP3 inflammasome through binding to toll-like receptor (TLR)4 and activating the NF-κB signalling pathway, NF-κB translocates into the nucleus to upregulate the expression of pro-IL-1β and NLRP3. LPS can also act as a PAMP via TLR4 by shifting metabolism in the cell to glycolysis and producing the metabolite, succinate, which activates the transcription factor, hypoxia inducible factor (HIF)1α, which also upregulates expression of pro-IL-1β and NLRP3. ATP acts as a DAMP for the second signal by inducing efflux of K^+ by triggering the ATP dependent K^+ channel P2X7. mROS produced by damaged mitochondria can also act as a DAMP for the second signal. Upon detection of the second signal, the NLRP3 components assemble, cleaving pro-IL-1β and pro-IL-18 to form IL-1β and IL-18 respectively, which are then subsequently secreted from the cell. Figure produced using Smart Servier Medical Art.

In recent years, immunometabolism has become an area of acute interest in the field of immunology, with several pathways identified as being involved in regulation of the NLRP3 inflammasome [425]. Under resting conditions cells produce ATP, via oxidative phosphorylation (OXPHOS) via the electron transport chain (ETC) in the mitochondria, with the Krebs cycle upstream

of this. OXPHOS is a highly efficient, but time-consuming pathway, whereby 34 ATP per one glucose molecule are produced; however, upon cellular stress, such as an immune cell reacting to a PAMP or DAMP, cellular metabolism switches to glycolysis. Glycolysis is a much more rapid but inefficient way for cells to generate ATP, as only 2 ATP are produced for every glucose molecule, which enables immune cells to react quickly to new and imminent threats, such as bacterial infection.

Everts *et al.* showed, in 2012, that stimulation of TLRs on monocyte-derived dendritic cells shift the cells to aerobic glycolysis, with a decline in OXPHOS production. This was found to be due to NO production and its inhibitory effect on OXPHOS, switching the cell to glycolysis and inducing ETC complex 1 to produce mROS to augment the innate immune response [426].

Several metabolites have also been shown to induce highly interesting immune responses, upon LPS challenge. Succinate production in macrophages after LPS stimulation, for example, has been linked to an elevated inflammatory gene expression profile, with succinates inhibition resulting in an anti-inflammatory outcome [427, 428]. This inflammatory gene profile was found to be dependent on the transcription factor, HIF-1α, as an increase in succinate leads to increased ETC complex 1 mROS production, which activates HIF-1α nuclear translocation and IL-1β expression [429, 430]. Another intermediate metabolite, itaconate, has been found to have anti-inflammatory properties, via its modification of cysteine residues on KEAP1, with dissociation of KEAP1 from Nrf2, thereby promoting Nrf2s translocation into the nucleus and upregulation of anti-oxidant and anti-inflammatory genes [431]. Glycolytic enzyme, hexokinase 1, the first enzyme in the glycolytic pathway, has also been found to be a key regulator of the NLRP3 inflammasome, as it directly interacts with NLRP3, leading to NLRP3 inflammasome activation and IL-1β secretion [432].

5.2 Results

5.2.1 Expression of Nrf2 system genes and proteins in CF monocytes and M1 macrophages

As no research has been carried out on the Nrf2 pathway in CF patient primary cells and the obvious links between p62 and Nrf2 function (see Figure 5.1), I decided to test the hypothesis that the Nrf2 pathway is dysregulated in CF monocytes and M1 macrophages, and may be partly responsible for the hyperinflammatory state we see in CF.

The expression of genes involved in the Nrf2 pathway was first measured using qPCR in HC and CF monocytes, both under basal conditions, and with LPS (Figure 5.3). There was no significant difference between HC and CF monocytes under basal conditions, or with LPS. However, there was a significant reduction in expression of *HO-1* after LPS stimulation in the CF monocytes. After this, the protein expression of both Nrf2 and KEAP1 was measured, using western blotting (representative blots shown in Figure 5.4A), both under basal conditions and with LPS. There was no significant difference found between HC and CF monocytes under basal conditions, and with LPS stimulation, for both KEAP1 (Figure 5.4B) and Nrf2 (Figure 5.4C).

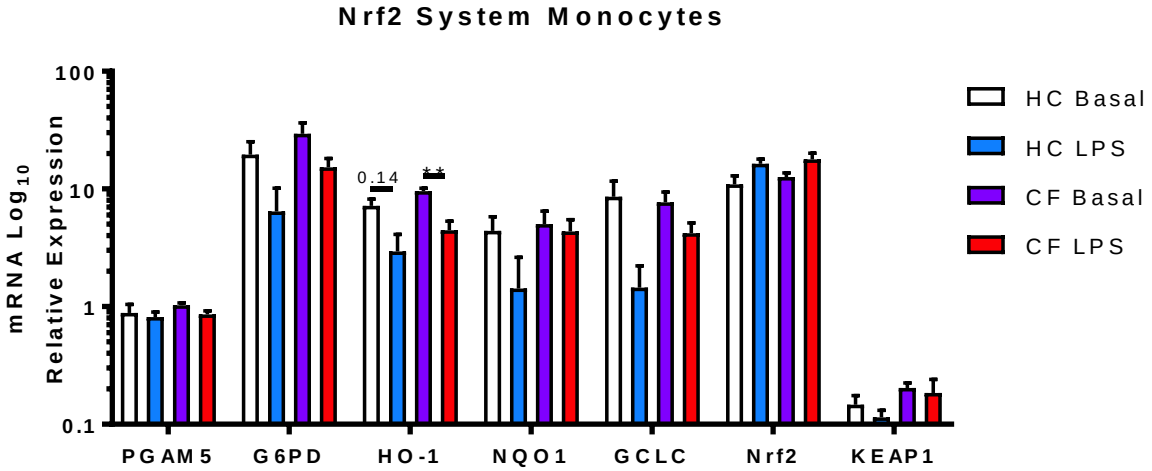


Figure 5.3 – Expression of Nrf2 system genes in monocytes. qPCR was used to measure the expression of Nrf2 system genes in both HC and CF monocytes. mRNA expression of *PGAM5*, *G6PD*, *HO-1*, *NQO1*, *GCLC*, *Nrf2* and *KEAP1* were measured under basal conditions and after 4 hours stimulation with 10 ng/ml LPS. All data are presented as mean ± SEM. Statistical significance was calculated using multiple t-test statistical analysis with Bonferroni-Dunn correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for HC and n=9 for CF, where n is the number of different HC or CF samples.

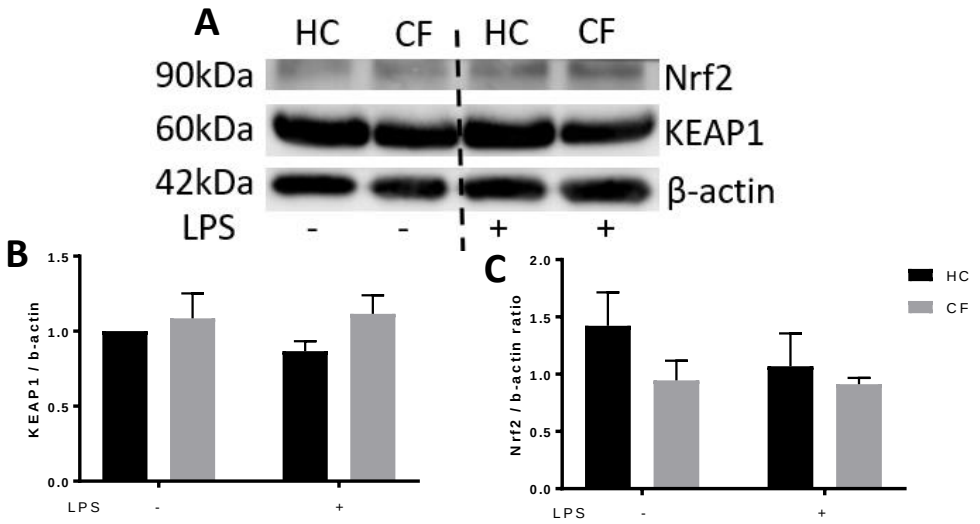


Figure 5.4 – Expression of Nrf2 and KEAP1 in monocytes. Western blots were used to measure the expression of Nrf2 and KEAP1 in both HC and CF monocytes under basal conditions and after 4 hours stimulation with 10 ng/ml LPS. Representative blots shown (A), protein expression of KEAP1 (B) and Nrf2 (C) was normalised to β-actin and the ratios were compared to HC monocytes. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8 for KEAP1 and n=6 for Nrf2, where n is the number of different HC or CF samples.

The expression of Nrf2 pathway genes was also investigated in M1 macrophages, under basal conditions, and after LPS stimulation using qPCR (Figure 5.5). There was no significant difference between HC and CF M1 macrophages, under basal conditions, or with LPS. Nrf2 and KEAP1 protein expression was also investigated using western blots in M1 macrophages under basal and LPS conditions, representative western blots shown in Figure 5.6A. There was no significance found across HC and CF for either condition for KEAP1 (Figure 5.6B) and Nrf2 (Figure 5.6C).

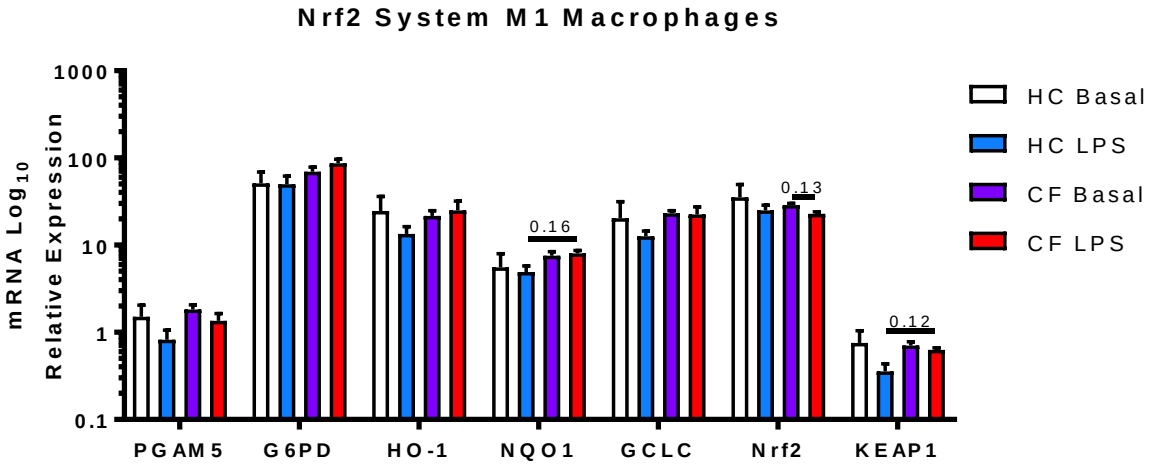


Figure 5.5 – Expression of Nrf2 system genes in M1 macrophages. qPCR was used to measure the expression of Nrf2 system genes in both HC and CF M1 macrophages. mRNA expression of *PGAM5*, *G6PD*, *HO-1*, *NQO1*, *GCLC*, *Nrf2* and *KEAP1* were measured under basal conditions and after 4 hours stimulation with 10 ng/ml LPS. All data are presented as mean ± SEM. Statistical significance was calculated using multiple t-tests statistical analysis with Bonferroni-Dunn correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=4, where n is the number of different HC or CF samples.

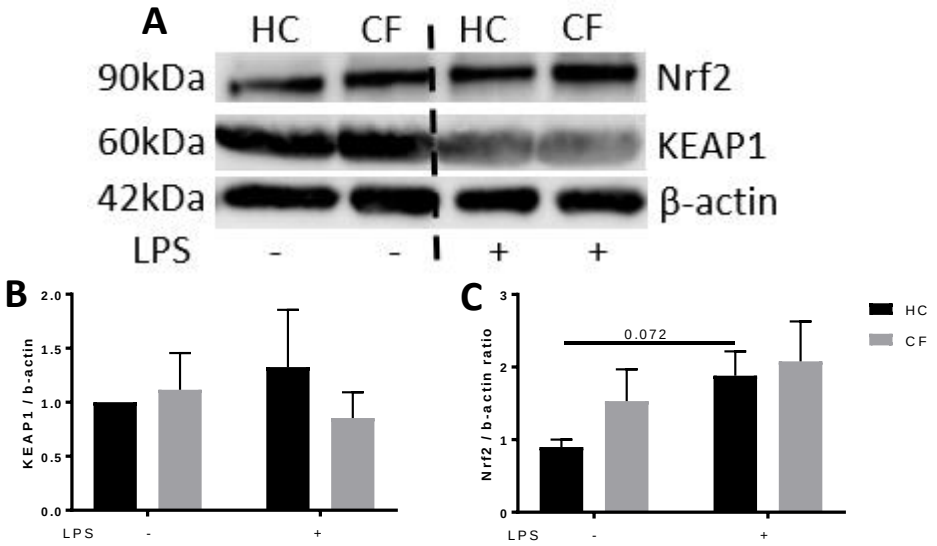


Figure 5.6 – Expression of Nrf2 and KEAP1 in M1 macrophages. Western blots were used to measure the expression of Nrf2 and KEAP1 in both HC and CF M1 macrophages under basal conditions and after 4 hours stimulation with 10 ng/ml LPS. Representative blots shown (A), protein expression of KEAP1 (B) and Nrf2 (C) was normalised to β-actin and the ratios were compared to HC M1 macrophages. All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 for KEAP1 and n=4 for Nrf2, where n is the number of different HC or CF samples.

5.2.2 Expression of mitophagy genes

As there were no significant differences found in the Nrf2 pathway, I decided to focus on another potential source of oxidative stress dysregulation, mitochondria. Expression of several genes, which are involved in clearance of damaged mitochondria from the cell via mitophagy, were investigated using qPCR in both HC and CF monocytes (Figure 5.7) and M1 macrophages (Figure 5.8) under basal conditions. Due to the low number of replicates statistical analyses were not performed

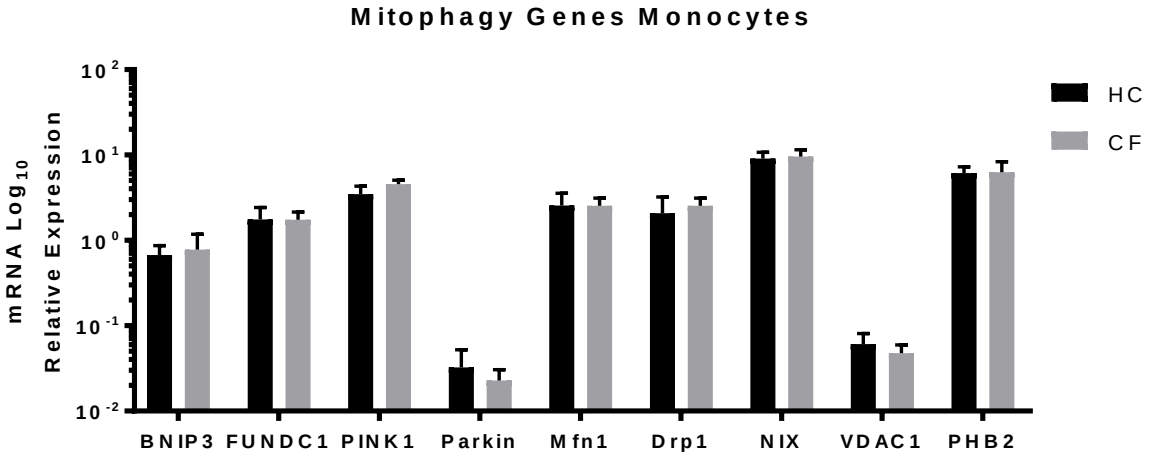


Figure 5.7 – Expression of mitophagy genes in monocytes. qPCR was used to measure the expression of mitophagy genes in both HC and CF monocytes. mRNA expression of *BNIP3*, *FUNDC1*, *PINK1*, *Parkin*, *Mfn1*, *Drp1*, *NIX*, *VDAC1* and *PHB2* were measured under basal conditions. All data are presented as mean ± SEM. n=3, where n is the number of different HC or CF samples.

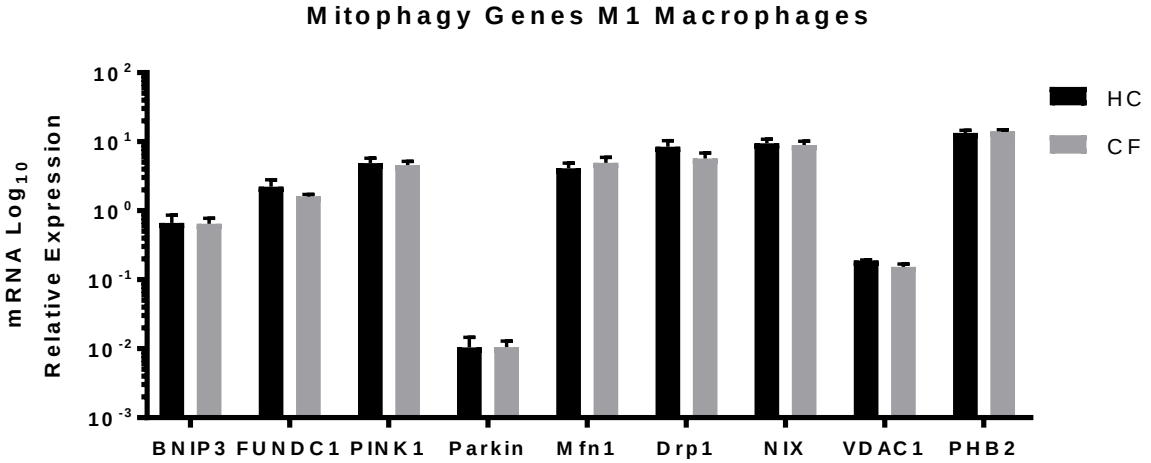


Figure 5.8 – Expression of mitophagy genes in M1 macrophages. qPCR was used to measure the expression of mitophagy genes in both HC and CF M1 macrophages. mRNA expression of *BNIP3*, *FUNDC1*, *PINK1*, *Parkin*, *Mfn1*, *Drp1*, *NIX*, *VDAC1* and *PHB2* were measured under basal conditions. All data are presented as mean ± SEM. Statistical significance was calculated using multiple t-tests statistical analysis with Bonferroni-Dunn correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=7 for HC and n=9 for CF, where n is the number of different HC or CF samples.

on the monocyte data (Figure 5.7). There was no significance found between HC and CF for the expression of any of these genes in M1 macrophages (Figure 5.8).

5.2.3 Expression of mitophagy and electron transport chain complex proteins

After finding no significant difference in gene expression of several mitophagy proteins in monocytes and M1 macrophages, I decided to focus on monocytes. This was because both CF monocytes and M1 macrophages appeared to function very similarly regarding autophagy/mitophagy and oxidative stress, this would allow me to investigate CF monocytes to a greater extent than if I continued with M1 macrophages at the same time.

Next the protein expression of TOMM40, COX4i1, PINK1 and Parkin were investigated in monocytes under basal conditions, representative blots shown in Figure 5.9A. There was no significance found between HC and CF monocytes (Figure 5.B).

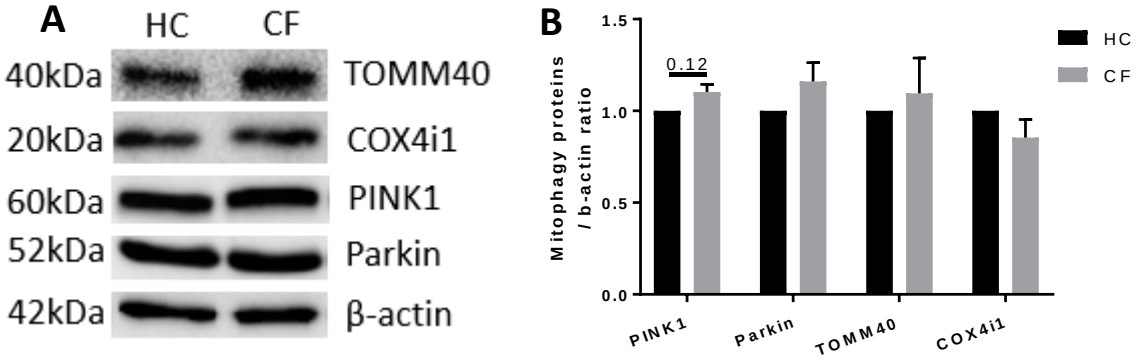


Figure 5.9 – Expression of mitophagy proteins in monocytes. Western blots were used to measure the expression of mitophagy proteins in both HC and CF monocytes under basal conditions. Representative blots shown (A), protein expression of PINK1, Parkin, TOMM40 and COX4i1 (B) was normalised to β-actin and the ratios were compared to HC monocytes. All data are presented as mean ± SEM. Statistical significance was calculated using multiple t-tests statistical analysis with Bonferroni-Dunn correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=6 and n=4 for TOMM40, where n is the number of different HC or CF samples.

Expression of the mitochondrial ETC complexes was also investigated, using western blots for both HC and CF monocytes, representative blots shown in Figure 5.10A. There was no significant difference found between HC and CF monocytes for any of the ETC complexes (Figure 5.10B).

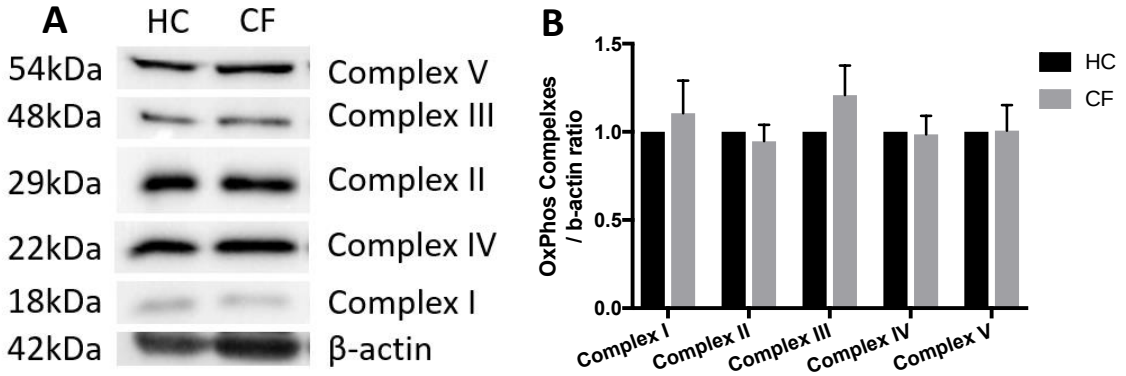


Figure 5.10 – Expression of mitochondrial electron transport chain proteins in monocytes. Western blots were used to measure the expression of mitophagy proteins in both HC and CF monocytes under basal conditions. Representative blots shown (A), protein expression of PINK1, Parkin, TOMM40 and COX4i1 (B) was normalised to β-actin and the ratios were compared to HC monocytes. All data are presented as mean ± SEM. Statistical significance was calculated using multiple t-tests statistical analysis with Bonferroni-Dunn correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8, where n is the number of different HC or CF samples.

5.2.4 Mitochondrial damage

As autophagy was dysregulated, indicated through the increased prevalence and lack of clearance of p62 in CF monocytes, I decided to investigate the state of mitochondria and whether dysregulated autophagy possibly leads to damaged or damage prone mitochondria.

First, I established the best way to induce and measure mitochondrial damage. I decided to try both low and high concentrations of LPS as well as trying the classic NLRP3 inflammasome activating signals, LPS and ATP in combination. To measure mitochondrial damage, I utilised flow cytometry with Mitotracker dyes Deep Red (MTDR) and Green (MTG), acquired from Invitrogen. MTDR stains functional/polarised mitochondria that have a stable mitochondrial membrane potential (ΔΨm), whereas MTG can stain all mitochondria if they are functional/polarised, or if they are damaged/depolarised. This means that, upon mitochondrial damage, MTDR (y axis of Figure 5.11A) will be unable to stain mitochondria, leading to a shift downwards towards MTG (x axis of Figure 5.11A). This allows for quantification of the percent of damaged mitochondria in the monocyte

population, as the more damaged the mitochondria are the higher the percent of mitochondria there are that are just stained with MTG.

Figure 5.11A shows representative MFIs for HC monocytes stained with MTDR and MTG under basal conditions, with LPS 10ng, LPS 50ng and LPS 10ng with ATP 5mM. Quantification of the percent of depolarised/damaged mitochondria in HC monocytes is shown in Figure 5.11B. Due to the low number of replicates, statistical analyses could not be performed. (Figure 5.11B).

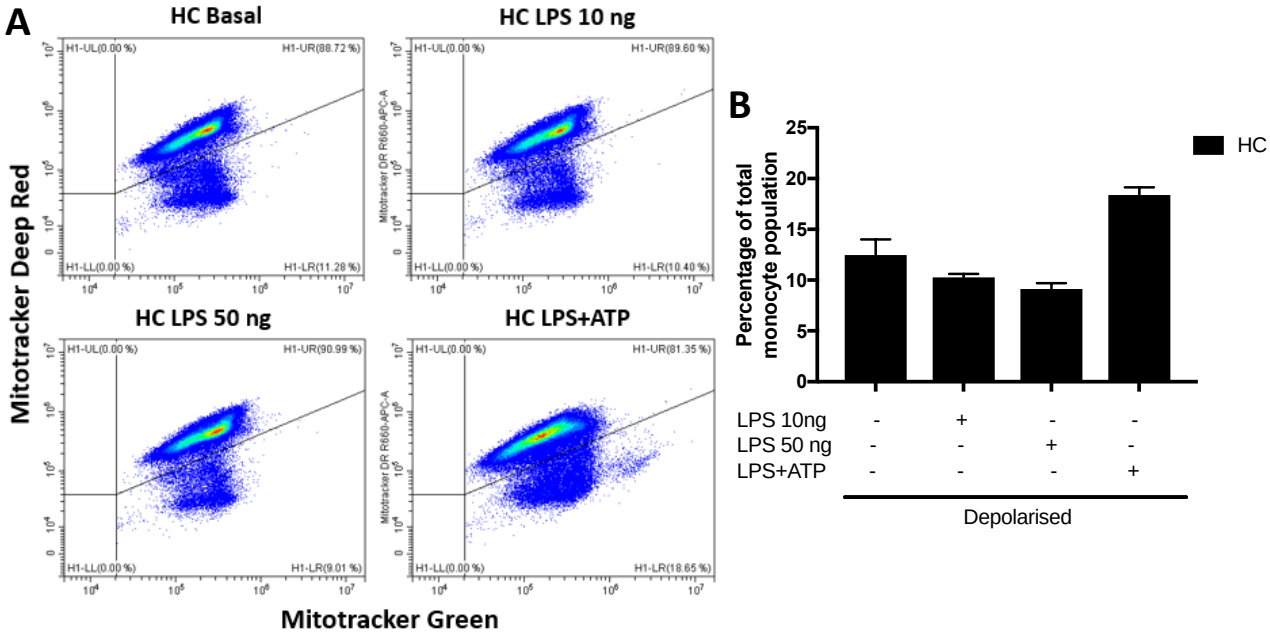


Figure 5.11 – Mitochondrial damage in HC monocytes. Flow cytometry was used to measure mitochondrial damage in HC monocytes under basal conditions and after 4 hours stimulation with LPS at 10 and 50 ng. 4 hours of LPs (10 ng) with 0.5 hours ATP (5mM) was also used. Respiring mitochondria were stained with Mitotracker Deep Red (MTDR) and all mitochondria were stained with Mitotracker Green (MTG), both dyes were acquired from Invitrogen. Gating strategy shown in Appendix Figure 3. Representative mean fluorescence intensity (MFI) (A) and percentage of damaged/depolarised mitochondria quantified (B). All data are presented as mean ± SEM. n=3, where n is the number of different HC or CF samples.

The percent of damaged mitochondria in CF monocytes was then compared to that of HC under basal conditions, and with LPS and ATP using flow cytometry, representative MFIs shown in Figure 5.12A. There was no significant difference under basal conditions between HC and CF monocytes in percent of damaged/depolarised mitochondria. However, there was a significant increase in percent of damaged mitochondria with LPS and ATP in CF monocytes compared to basal (Figure 5.12B).

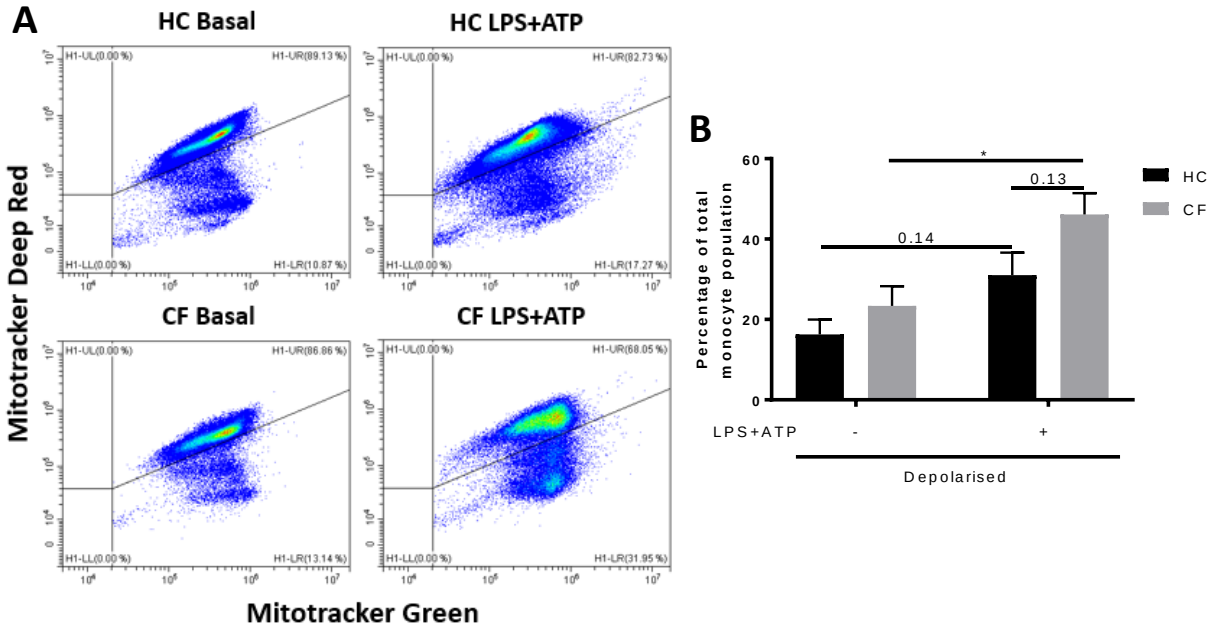


Figure 5.12 – Mitochondrial damage in monocytes. Flow cytometry was used to measure mitochondrial damage in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5mM) for the last 0.5 hours. Respiring mitochondria were stained with Mitotracker Deep Red (MTDR) and all mitochondria were stained with Mitotracker Green (MTG), both dyes were acquired from Invitrogen. Gating strategy shown in Appendix Figure 3. Representative mean fluorescence intensity (MFI) (A) and percentage of damaged/depolarised mitochondria quantified (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=10, where n is the number of different HC or CF samples.

5.2.5 Mitochondrial ROS production

Another measure of mitochondrial damage is production of mROS, and to measure this MitoSOX dye (Invitrogen) was utilised with flow cytometry; a representative MFI shown in Figure 5.13A. mROS production in HC and CF monocytes was measured under basal conditions, with LPS (10ng) and with LPS (10ng) and ATP (5mM) (Figure 5.13B). Under basal conditions, LPS and LPS with ATP there was a significant increase in production of mROS in CF monocytes compared to HC. There was also a significant increase in production of mROS in CF monocytes upon LPS and ATP stimulation, but not LPS on its own when compared to basal conditions.

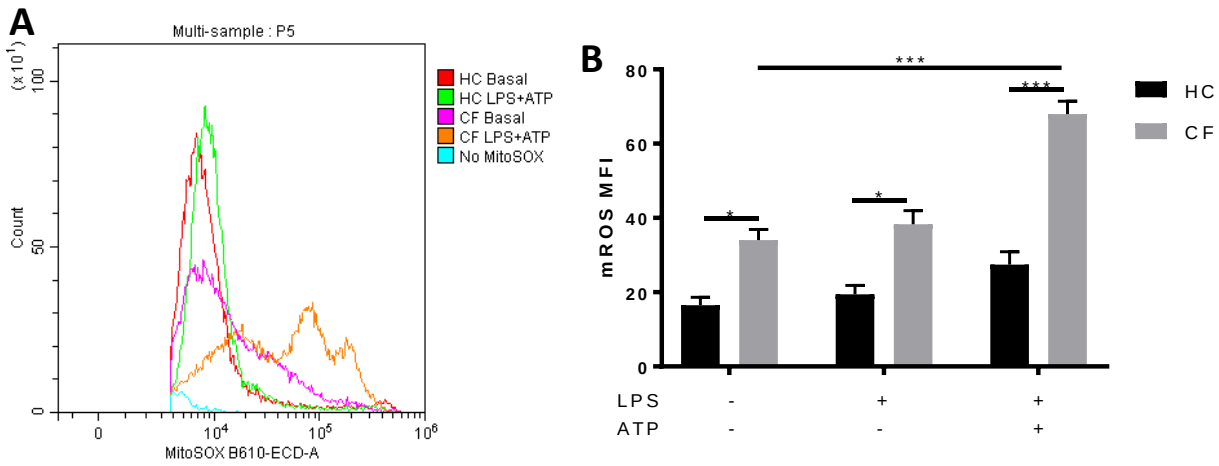


Figure 5.13 – Mitochondrial reactive oxygen species (mROS) production in monocytes. Flow cytometry was used to measure mROS production in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and LPS with the addition of ATP (5mM) for the last 0.5 hours. MitoSOX was used at 1 µM. Gating strategy shown in Appendix Figure 4. Representative mean fluorescence intensity (MFI) (A) and quantified MFI (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=5, where n is the number of different HC or CF samples.

5.2.6 Oxidative phosphorylation

Due to mitochondrial damage in CF monocytes, I decided to evaluate the state of OXPHOS in these cells, to establish whether damaged mitochondria in CF lead to metabolic abnormalities. To achieve this the metabolic analyser, Seahorse XF-96 Extracellular Flux Analyser (Agilent Seahorse Bioscience), was utilised with sequential addition of oligomycin, carbonyl cyanide-4-phenylhydrazone (FCCP) and rotenone/antimycin A to elicit various responses from mitochondria (Figure 5.14). Oxygen consumption rate (OCR) was used as an indicator of mitochondrial respiration to establish basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity. The first injection, oligomycin, prevents ATP production by inhibiting ATP synthase (complex V of the ETC), the reduction in OCR after addition of oligomycin is used to calculate ATP production. FCCP is then injected, disrupting ΔΨm and the proton gradient across the mitochondrial membrane but leaving the ETC uninhibited, allowing for maximal oxygen consumption via complex IV of the ETC. This increase in OCR is considered the maximal respiration and can be compared to ATP production and used to calculate spare respiratory capacity. The final injection is a combination of rotenone and antimycin A, which inhibit ETC complexes I and III, respectively, preventing mitochondrial respiration from taking place.

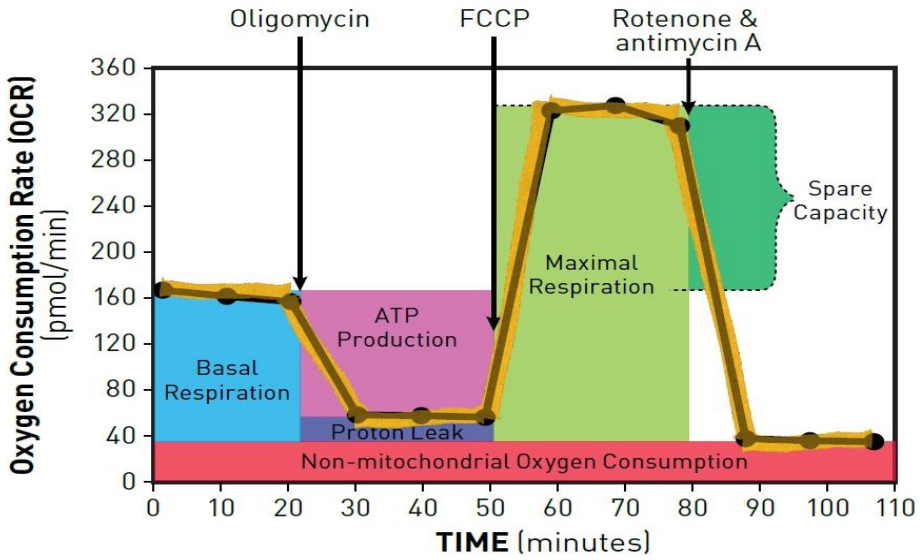


Figure 5.14 – Mitochondrial Respiration - Seahorse XF Cell Mito Stress Test Profile. Basal respiration, ATP production, proton leak, maximal respiration and spare respiratory capacity were measured. Oligomycin, carbonyl cyanide-4-phenylhydrazone (FCCP) and rotenone/antimycin A were used to inhibit various components of the mitochondria. Figure taken from Seahorse XF Mito Stress Report Generator.

The mitochondrial respiration of HC and CF monocytes was investigated under basal conditions, with LPS, and with LPS and ATP, representative OCRs shown in Figures 5.15A-C respectively. CF monocyte basal respiration and ATP production were significantly increased under basal conditions compared to HC (Figure 5.15A). With LPS and ATP, there was a significant increase in basal respiration, ATP production as well as proton leak between CF monocytes and HC. Proton leak showed a significant increase when comparing CF monocytes with LPS and ATP to LPS. Maximal respiration showed a significant reduction when comparing CF monocytes with LPS and ATP to both LPS and basal conditions. There was a significant reduction shown in spare respiratory capacity for CF monocytes, upon addition of LPS and ATP, compared to both LPS and basal conditions.

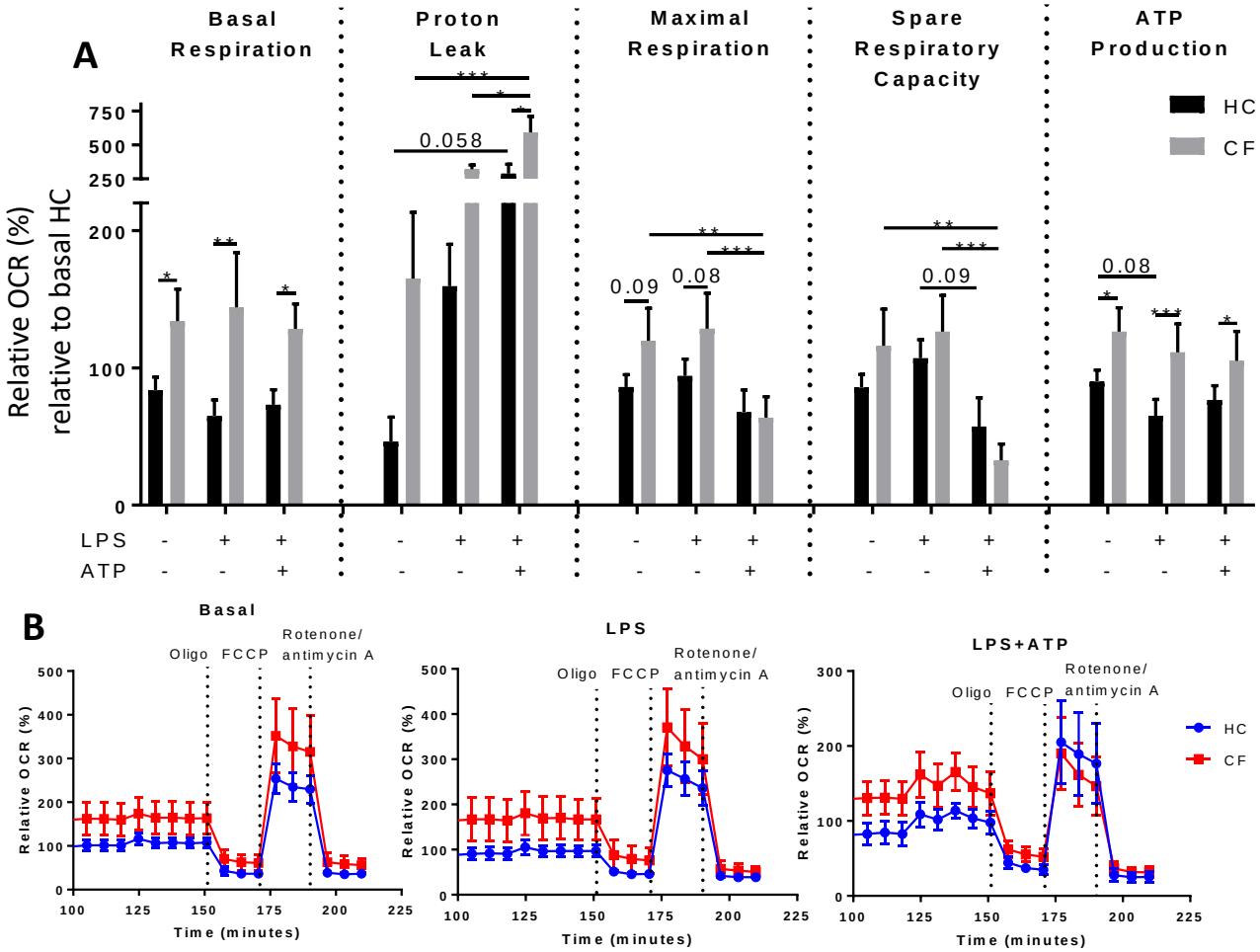


Figure 5.15 – Oxidative phosphorylation in CF monocytes. A seahorse XF-96 extracellular flux analyser was used to measure the real time oxygen consumption rate (OCR) in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5 mM) for the last 0.5 hours. Oligomycin used at 1 µM, FCCP at 1 µM and rotenone/antimycin A at 0.5 µM. Basal OCR, proton leak, maximal respiration, spare respiratory capacity and ATP production (A). Real time OCR (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8, where n is the number of different HC or CF samples.

5.2.7 Glycolysis

Extracellular acidification rate (ECAR) was also measured in the same experiment, this is used as a readout for glycolysis as measurement of pH changes correlates with amount of lactic acid being produced by the cell and therefore rate of glycolysis. Representative ECAR readouts for basal conditions, LPS and LPS with ATP shown in Figure 5.16A. Under basal conditions there is a significant

increase in ECAR in CF monocytes compared to HC, with a significant increase in ECAR upon addition of LPS for both HC and CF monocytes relative to basal conditions (Figure 5.16B). Upon addition of LPS the difference between CF and HC monocytes becomes even more significant. When LPS and ATP is added to the cells, there is a significant increase in ECAR for both HC and CF compared to basal conditions.

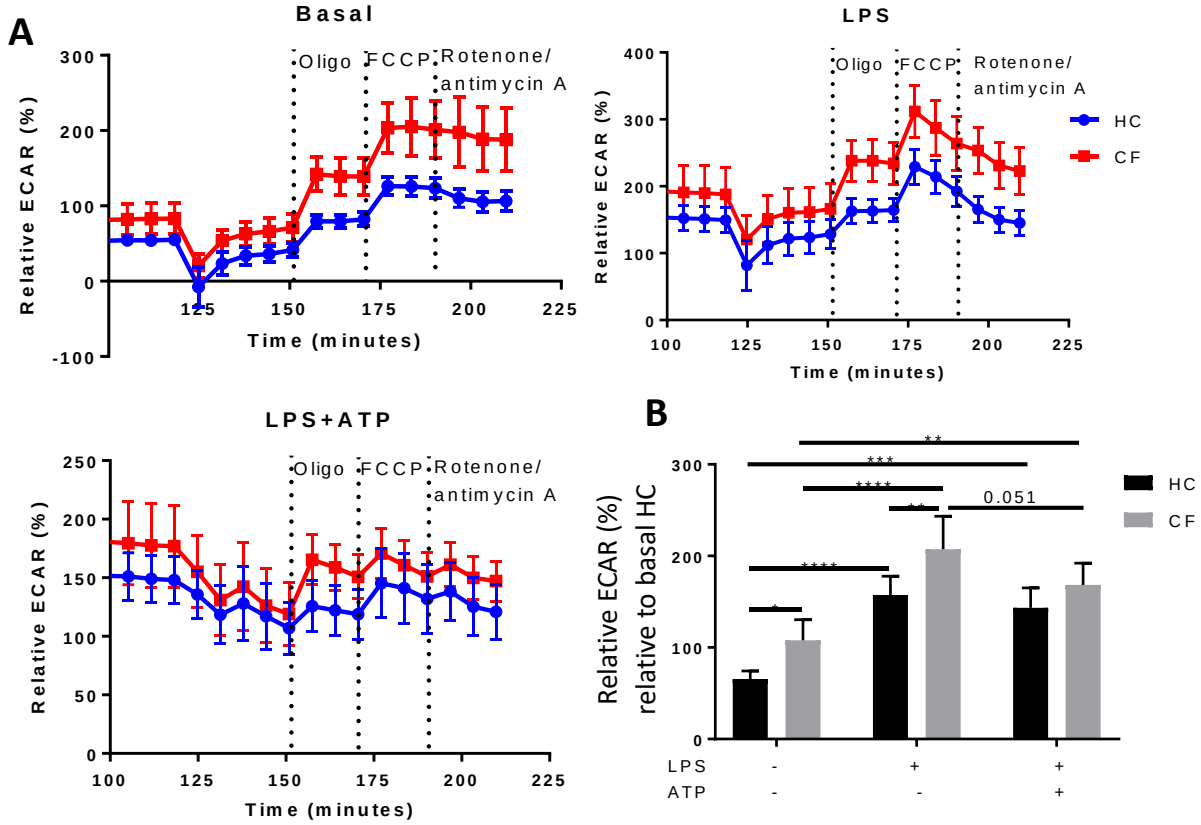


Figure 5.16 – Extracellular acidification rate in CF monocytes. A seahorse XF-96 extracellular flux analyser was used to measure the real time extracellular acidification rate (ECAR) in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5 mM) for the last 0.5 hours. Oligomycin used at 1 µM, FCCP at 1 µM and rotenone/antimycin A at 0.5 µM. Real time ECAR (A), quantification of average real time ECAR (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8, where n is the number of different HC or CF samples.

To further establish the rate of glycolysis in CF monocytes, Dr Thomas Scambler and Dr Heledd Jarosz-Griffiths measured ATP and L-lactate production, glucose consumption and succinate accumulation using colorimetric assays (Abcam) (unpublished data). ATP is measured here (Figure 5.17A) as the final product of both OXPHOS and glycolysis and is used as a marker for how metabolically active the cells are, as a greater amount of ATP would correlate with greater energy demands. In glycolysis, pyruvate is reduced by lactate dehydrogenase to form lactate (normally the L-lactate isomer), measuring L-lactate (Figure 5.17B) in extracellular fluid can be correlated with the rate of glycolysis. Glucose is the main substrate for ATP production for both OXPHOS and glycolysis, making measurement of its consumption (Figure 5.17C) an imperfect measurement of ATP production via glycolysis. However, as OXPHOS requires the products of glycolysis, namely pyruvate and acetyl-coenzyme A for the Krebs cycle, glucose consumption is a good measurement of glycolysis and therefore metabolic activity but not what pathway is being fed into. The Krebs cycle metabolite, succinate, was also measured (Figure 5.17D) as it is known to accumulate upon LPS stimulation, when the cell switches from OXPHOS to glycolysis for ATP production.

Due to the low n number, statistical analyses could not be performed on Figure 5.17.

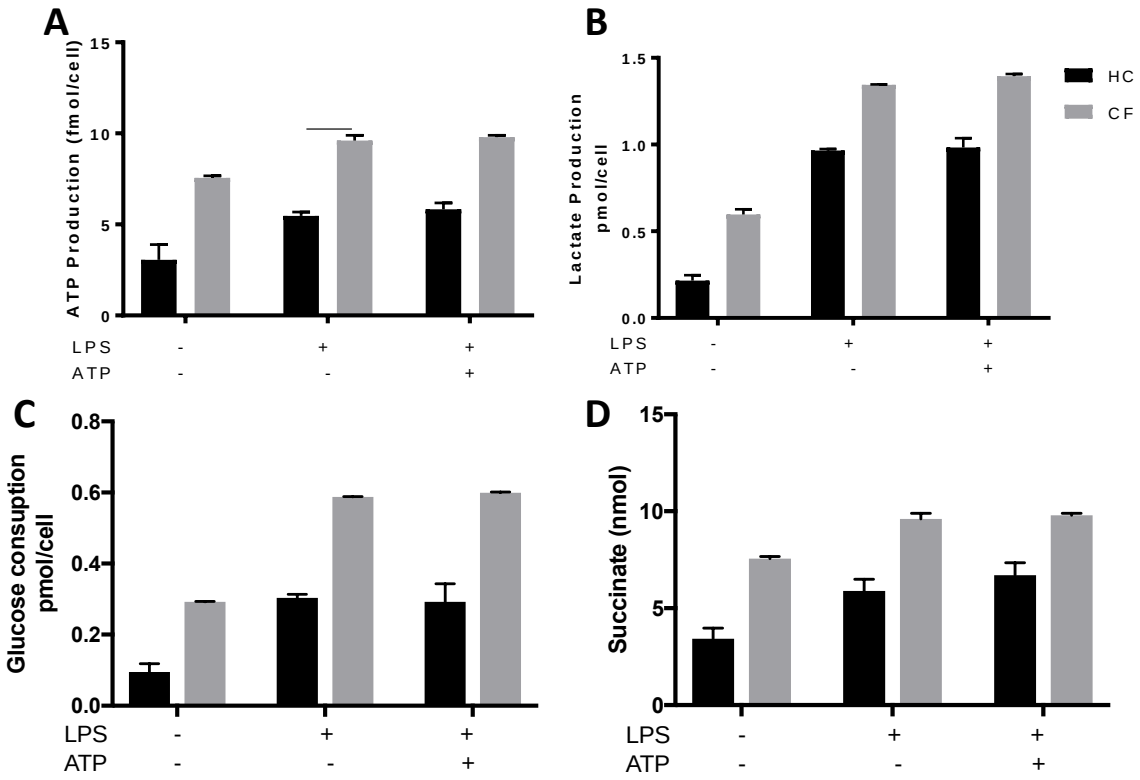


Figure 5.17 – Glycolysis in monocytes. ATP and L-lactate production, glucose consumption and succinate accumulation were measured using colorimetric assays (all Abcam) in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5 mM) for the last 0.5 hours. Data provided by Dr Thomas Scambler and Dr Heledd Jarosz-Griffiths, unpublished data. All data are presented as mean ± SEM. n=3, where n is the number of different HC or CF samples.

5.2.8 Mitochondrial damage with CFTR inhibition

To investigate the link between CFTR lack of function and increased damaged mitochondria the CFTR inhibitor, R enantiomer (R) benzopyrimido-pyrrolo-oxazinedione (BPO)-27 (MedChemExpress), was utilised with flow cytometry and Mitotracker dyes, MTDR and MTG. A comparison was made between HC monocytes, with and without the CFTR inhibitor, (R) BPO-27. (R) BPO-27 was used at 100 nM for 2 hours, as previous publications had reported its ability to inhibit CFTR effectively at this concentration and time [433]. I decided to avoid the commonly used CFTR inhibitor $CFTR_{inhib}$-172, due to its reported ability to alter mitochondrial function, independently of its inhibition of CFTR function [301].

The same flow cytometry assay utilised in Figure 5.12 was used, with representative MFIs shown in Figure 5.18A. Under basal conditions, there was no significant difference between HC monocytes with and without (R) BPO-27, and the same was found after addition of LPS (Figure 5.18B). Upon stimulation with LPS and ATP, there was a significant increase in the percentage of damaged mitochondria in HC monocytes, both with and without (R) BPO-27, with no significant differences found between the HC samples with or without the inhibitor.

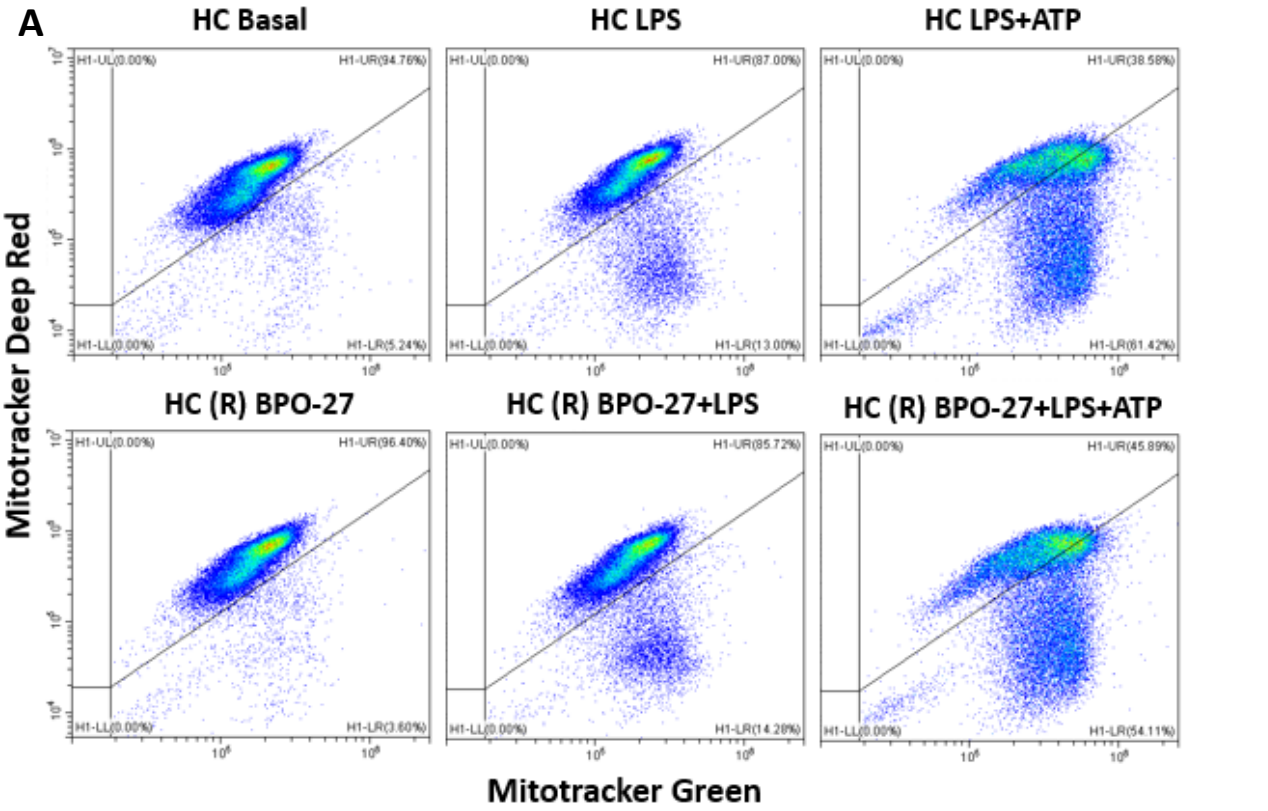


Figure 5.18 – Mitochondrial damage in HC monocytes with CFTR inhibitor. Flow cytometry was used to measure mitochondrial damage in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5mM) for the last 0.5 hours. (R) BPO-27 was used at 100 nM for 2 hours to inhibit the CFTR. Respiring mitochondria were stained with Mitotracker Deep Red (MTDR) and all mitochondria were stained with Mitotracker Green (MTG), both dyes were acquired from Invitrogen. Gating strategy shown in Appendix Figure 3. Representative mean fluorescence intensity (MFI) (A) and percentage of damaged/depolarised mitochondria quantified (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=4, where n is the number of different HC or CF samples.

5.2.9 Mitochondrial ROS production with CFTR inhibition

MitoSOX was also utilised to measure mROS production, with and without (R) BPO-27; representative MFIs from the flow cytometry assay are shown in Figure 5.19A. Under basal conditions there was no significant difference found between HC monocytes with or without the CFTR inhibitor (Figure 5.19B). Upon addition of LPS, there was a significant increase in mROS production, compared to basal, and with LPS and ATP there was an even more significant increase, for both HC monocytes with and without (R) BPO-27. There was no significant difference found between HC monocytes with and without (R) BPO-27.

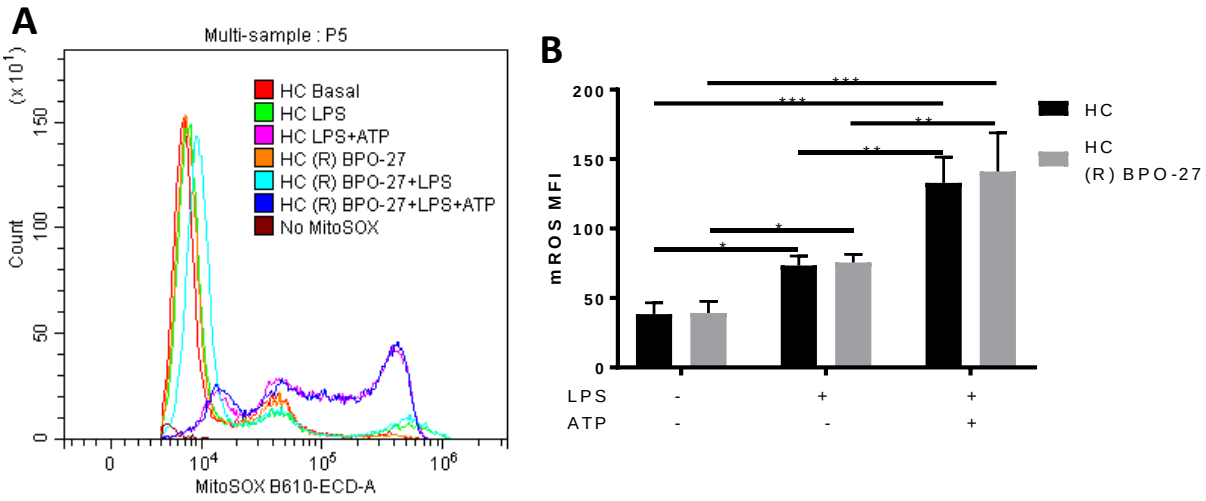


Figure 5.19 – Mitochondrial reactive oxygen species (mROS) production in HC monocytes with CFTR inhibitor. Flow cytometry was used to measure mROS production in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and LPS with the addition of ATP (5mM) for the last 0.5 hours. (R) BPO-27 was used at 100 nM for 2 hours to inhibit the CFTR. MitoSOX was used at 1 µM. Gating strategy shown in Appendix Figure 4. Representative mean fluorescence intensity (MFI) (A) and quantified MFI (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=4, where n is the number of different HC or CF samples.

5.2.10 Oxidative phosphorylation with CFTR inhibition

The impact inhibition of CFTR function with (R) BPO-27 was then investigated, to establish whether there was a link between CFTR function in monocytes and metabolism. The same Seahorse assay was performed as in Figure 5.15, with representative OCR readouts for basal, LPS, and LPS with ATP shown in Figure 5.20B. Under basal conditions, with LPS, and with LPS and ATP, there was no significant change in basal respiration or ATP production for both HC monocytes with and without (R) BPO-27 (Figure 5.20A). For proton leak, there was no significant difference between HC with and without (R) BPO-27 across any of the stimulations. There was a significant reduction in maximal respiration under basal conditions for HC monocytes with (R) BPO-27 compared to HC under the same conditions. Upon addition of LPS, there was a significant increase in maximal respiration for the HC monocytes with (R) BPO-27 but not the monocytes without (R) BPO-27. When LPS and ATP were added, there was a significant reduction in maximal respiration compared to basal conditions for HC monocytes with (R) BPO-27. Spare respiratory capacity showed no significant difference between HC monocytes with and without (R) BPO-27. When stimulated with LPS, there was a significant increase in spare respiratory capacity for HC monocytes with (R) BPO-27 but not without the inhibitor. With LPS and ATP there was a significant decrease in spare respiratory capacity for HC monocytes without (R) BPO-27 compared to baseline, there was no difference with the inhibitor.

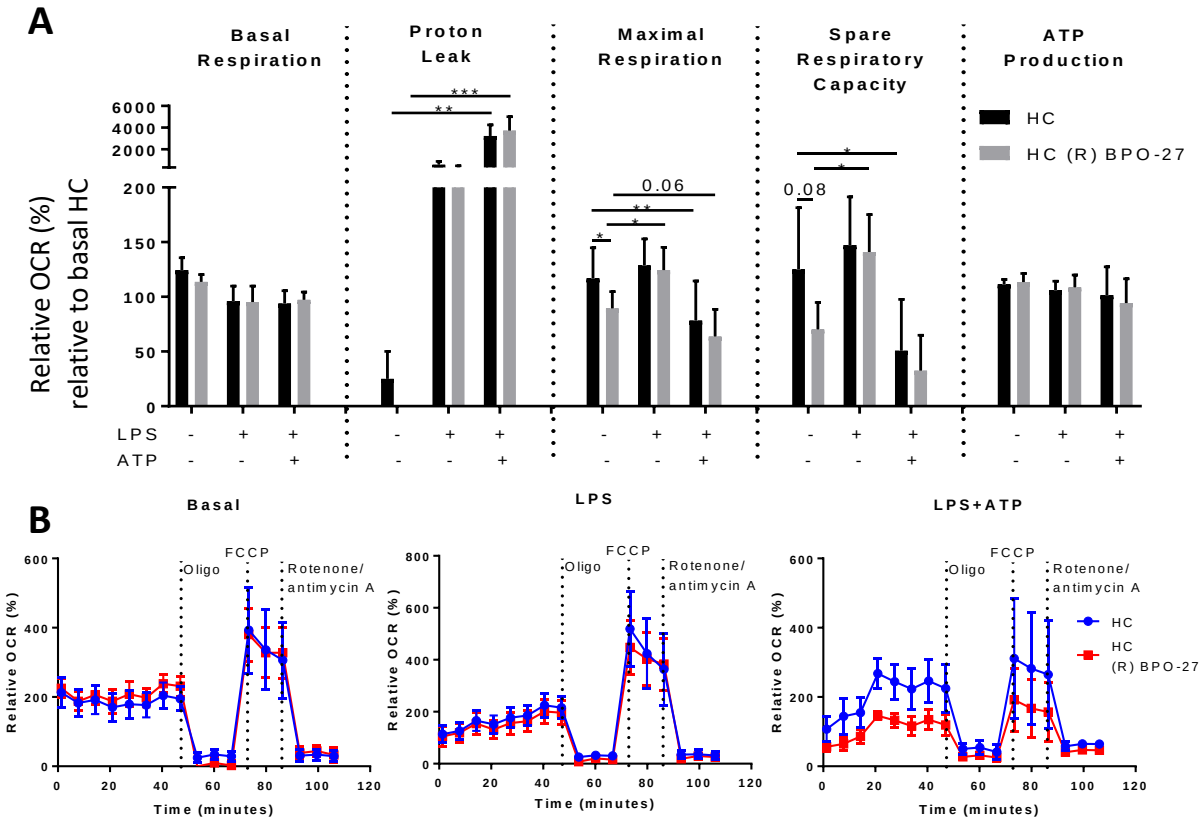


Figure 5.20 – Oxidative phosphorylation in HC monocytes with CFTR inhibitor. A seahorse XF-96 extracellular flux analyser was used to measure the real time oxygen consumption rate (OCR) in HC monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5 mM) for the last 0.5 hours. (R) BPO-27 was used at 100 nM for 2 hours to inhibit the CFTR. Oligomycin used at 1 µM, FCCP at 1 µM and rotenone/antimycin A at 0.5 µM. Basal OCR, proton leak, maximal respiration, spare respiratory capacity and ATP production (A). Real time OCR (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=4, where n is the number of different HC or CF samples.

5.2.11 Glycolysis with CFTR inhibition

ECAR was also measured in the same experiment, representative ECAR readouts shown in Figure 5.21A. There was no significant difference found between HC monocytes with or without the (R) BPO-27 under basal conditions, with LPS, or with LPS and ATP (Figure 5.21B). However, there was a significant increase in ECAR upon addition of LPS and ATP for HC monocytes without (R) BPO-27, but not with (R) BPO-27.

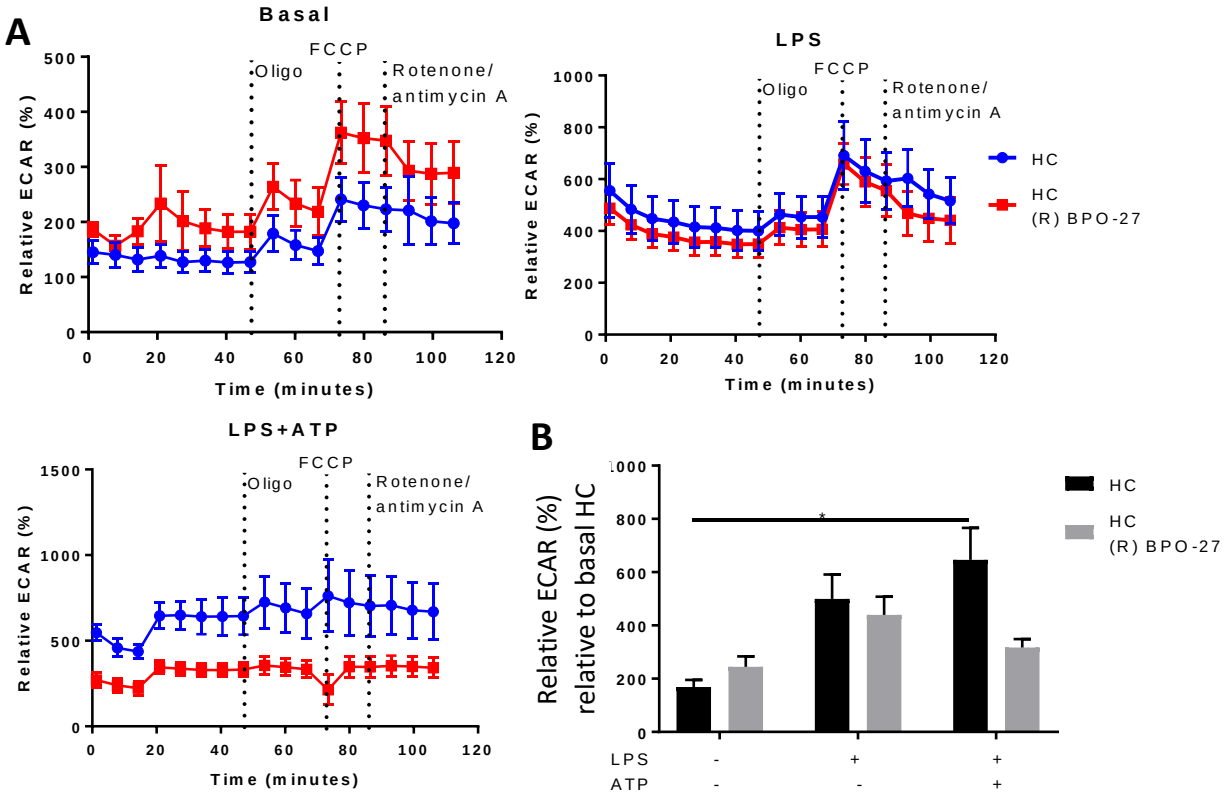


Figure 5.21 – Extracellular acidification rate in HC monocytes with CFTR inhibitor. A seahorse XF-96 extracellular flux analyser was used to measure the real time extracellular acidification rate (ECAR) in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5 mM) for the last 0.5 hours. (R) BPO-27 was used at 100 nM for 2 hours to inhibit the CFTR. Oligomycin used at 1 µM, FCCP at 1 µM and rotenone/antimycin A at 0.5 µM. Real time ECAR (A), quantification of average real time ECAR (B). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=4, where n is the number of different HC or CF samples.

5.3 Discussion

5.3.1 Oxidative stress in CF

Oxidative stress and Nrf2 dysregulation are well-established characteristics of CF airway epithelial cells [410, 411] with up to a 70% reduction in expression of Nrf2 [410]. In contrast to this, based on data in this chapter, the Nrf2 pathway is largely not dysregulated in both CF monocytes and M1 macrophages under basal conditions, or with LPS as a stimulant. The only significant difference found between HC and CF monocytes in the Nrf2 pathway was significantly reduced gene expression of HO-1 after LPS stimulation compared to basal conditions, as there was no such reduction in HO-1 expression in HC monocytes after LPS stimulation. This could potentially indicate that in response to bacterial infection the inflammatory response is less restrained, due to the increased presence of HO-1 substrates (heme and biliverdin) which are not broken down into their anti-inflammatory forms (CO and bilirubin). This dysregulation of HO-1 could add to the hyperinflammatory state seen in CF [28, 103], due to CO, bilirubin's and HO-1's anti-inflammatory and anti-oxidant properties [398-401]. There was no change in protein expression of Nrf2 in HC and CF M1 macrophages after LPS stimulation; this should be tested again with a larger n number to elucidate if a significant difference exists.

It was a surprising result to find no significant difference in protein expression of KEAP1 and Nrf2 as the expression of p62 was significantly raised under basal conditions in CF monocytes and M1 macrophages. As p62 binds to KEAP1 under cellular stress, targeting it for degradation via autophagy, I expected KEAP1 to be raised in CF under basal conditions relative to HC due to the lack of autophagic flux shown in the previous chapter. This is coupled with the fact that the Nrf2 pathway has been shown to play a protective role against pulmonary fibrosis in both a mouse model and HBECs [434].

5.3.2 Mitochondrial damage in CF monocytes

There was no significant difference found in the expression of several mitophagy genes and proteins; however, the mitochondrial association of these mitophagy proteins may be abnormal, so western blots would need to be performed on isolated mitochondria to establish whether p62, PINK1

and Parkin are associating with the mitochondria correctly in CF. Furthermore, no significant difference was found between HC and CF monocytes in protein expression of the ETC complexes, implying CF monocytes have the same number of mitochondria as HC. However, as ETC I and III are the largest producers of mROS it might be expected that these could be overexpressed in CF due to overproduction of mROS.

Mitochondrial depolarisation has been shown in the CF IB3-1 HBEC line relative to the C38 "WT" cell line [79], and was linked to dysregulated autophagy. Based on data presented in this chapter, I can state that the mitochondria in monocytes, which are homozygous for ΔF508, are more damaged and are more prone to damage upon stimulation. This is since the mitochondria in CF monocytes produce significantly more mROS under basal conditions, as well as with LPS and ATP stimulation compared to HC, in addition to the observation that CF monocytes are more depolarised upon LPS and ATP stimulation compared to HC.

Given that mitochondria are damaged and more prone to damage in CF monocytes, it is likely that this contributes to the inflammatory phenotype found in CF and may contribute to dysregulated autophagy as well. As the current model of dysregulated autophagy in CF depends on significantly increased levels of ROS upstream of dysregulated autophagy [79]. Furthermore, mROS has been reported to be able to induce NLRP3-dependent lysosomal damage in BMDMs of mice, leading to lysosomal membrane permeabilisation and deacidification, this effect was significantly reduced upon use of mROS scavenger mitoTEMPO [435]. A potentially useful biomarker for inflammatory diseases, like CF, which are known to have mitochondrial damage as part of their phenotype, would be the measurement of serum levels of mtDNA or small extracellular vesicles with a specific mitochondrial signature [436, 437]. This could potentially be a useful indicator as to the presence of mitochondrial damage.

Binding of ATP to the K^+ channel, P2X7, has been shown to lead to mitochondrial damage due to an influx of Ca^{2+} depolarising the mitochondria [318, 320]. So it is likely that given the ionic

imbalances already reported in CF monocytes [28], raised levels of intracellular Ca^{2+} in CF neutrophils [40], CF PBMCs [438], CF lymphocytes [439], and in the CF IB3-1 cell line [121], there may be raised intracellular Ca^{2+} contributing to the damaged mitochondrial phenotype seen in CF monocytes. In addition to this, the important mediator of innate inflammation, sTREM-1, which has been reported to be raised in CF [87, 88], is able to induce the rapid mobilisation of Ca^{2+} [86, 440], which may also contribute to any increased intracellular Ca^{2+} concentration found in CF. Furthermore, Ca^{2+} movement, upon ATP stimulation, has been shown to be critical to inducing mitochondrial damage and inducing NLRP3 inflammasome mobilisation [318, 319]. Na^{+} have also been shown to contribute to mitochondrial depolarisation [441].

The Ca^{2+} ion channel blocker, amiodarone, has been reported to increase autophagic markers in the CF HBEC line, Cfbe41o-, with further increases upon stimulation with cysteamine as well [352]. The same study also found that amiodarone was able to improve ΔF508 CFTR membrane retention in Cfbe41o-. However, the mechanism by which amiodarone achieves this is unclear, but it was suggested to perhaps be due to its upregulation of autophagy [352]. One recent study provided a possible explanation for this, as an increase in cytosolic Ca^{2+} has been found to induce dynamin and calcineurin-dependent internalisation of CFTR, the inhibition of Ca^{2+} channels would result in decreased cytosolic Ca^{2+}, and therefore, increased CFTR cell membrane retention [38]. This link between increased cytosolic Ca^{2+} and reduced CFTR membrane expression was initially found to be induced by cigarette smoke (CS) [442]. Increased cytosolic Ca^{2+} concentration, via dysregulation of calcium selective ion channel, Orai1, has also been linked to increased IL-8 production in CF [443].

Another explanation for an increase in damaged mitochondria is the protein kinase, CK2, which has been found to be overactive in CF [53]. As CK2 has been reported to regulate the mitophagy protein, FUNDC1, by phosphorylating it and effectively downregulating its activity, the overactivity of CK2 may result in a downregulation in FUNDC1, leading to reduced rate of mitophagy [261].

Inflammatory signals, such as LPS and ATP, have been shown to induce autophagy at the same time as inducing activation of the NLRP3 inflammasome [218]. So, once the NLRP3 inflammasome is activated, negative feedback loops are already at work restraining its activation. As autophagy is dysregulated in CF monocytes, this important regulation of the NLRP3 inflammasome is defective and results in excessive inflammation. NF-κB has been found to be upregulated in CF and plays a key role in promoting the inflammatory phenotype in CF; however, NF-κB has also been reported to be involved in anti-inflammatory processes in macrophages, such as the clearance of damaged mitochondria via upregulation of p62 [367]. As there is a build-up and lack of clearance of p62 in both CF monocytes and M1 macrophages, it is possible that NF-κB dysregulation contributes to this p62 accumulation.

The mitophagy inducer, p62-mediated mitophagy inducer (PMI), has been shown to induce mitophagy independently of ΔΨm collapse and could, therefore, be an interesting avenue of future research to establish if inducing clearance of damaged mitochondria in CF results in a reduction in NLRP3 inflammasome activation. PMI is unique to other mitophagy inducers, such as urolithin A and acitonin, in that it doesn't damage mitochondria to induce mitophagy. Instead it induces the Nrf2 pathway via directly binding to Nrf2s regulator, KEAP1, driving the non-canonical Nrf2 pathway and resultant anti-inflammatory and anti-oxidant response [444]. PMI has also been reported to increase poly-ubiquitination of mitochondria and promote localisation of autophagy protein, LC3, to mitochondria, increasing the clearance of damaged mitochondria [444]. Another small molecule found to induce mitophagy, berberine (BBR), has been reported to reduce lung inflammatory injury, release of ROS, as well as inhibiting release of MCP-1 and TNF in mice infected with influenza [445]. BBR has also been shown to inhibit NLRP3 inflammasome activation in a mouse macrophage cell line (J774.1) by decreasing mROS and increasing ΔΨm via mitophagy [446]. Furthermore, BBR has been shown to act via PERK of the UPR to upregulate the Nrf2 pathway and ameliorate LPS induced acute lung injury [447]. Due to these molecules beneficial effects in clearing damaged mitochondria, resolving inflammation and ameliorating acute lung injury, they may be of therapeutic interest for

treatment of CF. As their ability to induce mitophagy without damaging the mitochondria has already been published, it would be interesting to establish what impact these molecules have on metabolism.

5.3.3 Metabolism in CF monocytes

It is clear from the data presented in this chapter that CF monocytes have an increased metabolic phenotype, with raised basal respiration and ATP production through OXPHOS, but also raised glycolysis, indicated by a significantly increased ECAR under basal conditions compared to HC. Also, as protein expression of the ETC complexes is the same between HC and CF monocytes, the rate of OXPHOS has increased in CF monocytes despite having the same quantity of mitochondria. Not only are OXPHOS and glycolysis raised under basal conditions but upon LPS challenge there is a heightened sensitivity to switch to glycolysis, meaning a greater inflammatory response upon stimulation. An increased metabolic state, metabolic reprogramming and heightened inflammation has already been reported in M1 macrophages in CF, with hyperactivation of the IRE1α-XBP1 pathway of the UPR being one of the main pathways contributing to this [103]. Another paper has also shown this metabolic switch in CF neutrophils, with increased secretion of IL-1β and its subsequent inhibition upon use of 2-deoxy-D-glucose (2DG), an inhibitor of glycolysis, emphasising the link between the metabolic switch and inflammation [92]. This shift to glycolysis upon LPS challenge has been shown, in macrophages by several reports, to drive inflammation [427, 429, 448]. A recent study demonstrated that neutrophils in both a CF mouse model and patients with CF, retain an increased glycolytic state with increased NLRP3 inflammasome activation. Upon specific inhibition of the NLRP3 inflammasome, with MCC950, they found reduced IL-1β production with improved clearance of *P. aeruginosa* in the lungs of CF mice [185].

This heightened energy demand could be linked to the increased activity of ENaC, leading to an increase in intracellular Na^+ and decreased intracellular K^+, this might result in the activation of the Na^+/K^+ channel with ATP to restore ionic homeostasis [28, 449]. This process requires considerable

energy to try and maintain ionic homeostasis, and therefore drives up the energy demand of the cell with increased OXPHOS and glycolysis to fuel increased demand.

Given that proton leak is significantly increased upon addition of LPS and ATP in CF monocytes relative to HC, it indicates that mitochondrial membranes in CF monocytes become more compromised by this stimulation than it does in HC monocytes, showing that mitochondria in CF are more prone to damage, and therefore, more prone to inducing an NLRP3 driven inflammatory response. This may be responsible for the significant reduction in maximal respiration seen in CF monocytes, and therefore, the spare respiratory capacity as well, despite ATP production still being significantly raised.

Increased ECAR indicated heightened glycolysis, to further establish this observation, other glycolytic markers were investigated; however, more patient samples are required before statistical analyses can be performed, and any conclusions drawn.

Raised levels of succinate have been previously linked to a downregulation of the central regulator of metabolism, PTEN, as the CFTR mutation has been shown to result in diminished PTEN activity in human epithelial cell line, HTC116, and HBEC line 16HBE. This was found to promote the colonisation of the airway by *P. aeruginosa*, as the bacteria metabolise succinate, leading to an anti-inflammatory host response [303]. PTEN may also be downregulated in CF monocytes and be partially responsible for the altered metabolic state described.

Increased cytosolic and mitochondrial Ca^{2+} levels have been reported to drive ATP production, as Ca^{2+} modulates Krebs cycle enzymes, pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase [321, 450-452]. With intracellular ionic imbalances already established (Na^{+} and K^{+}) in CF monocytes [28], it is likely that there are other intracellular ionic imbalances, such as Ca^{2+}. Raised intracellular Ca^{2+} levels have already been shown in the IB3-1 CF HBEC line, and has been linked to increased activation of NF-κB and raised inflammation [121]. Given that primary neutrophils, taken from ΔF508 homozygous CF patients, have been found to have raised intracellular

Ca^{2+} concentrations [40], it is likely that monocytes with the same mutations would have similar intracellular Ca^{2+} levels. Furthermore, increased intracellular Na^{+} and decreased K^{+} have been shown in CF monocytes [28], and through P2X7 has been found to increase mitochondrial Ca^{2+} levels [320]. In all, this makes it likely that raised intracellular Ca^{2+} levels contribute to the heightened metabolic state seen in CF monocytes.

5.3.4 Mitochondrial damage and metabolism link to CFTR function

To establish what role the lack of a functional CFTR protein plays in damaged mitochondria and heightened metabolic state found in CF monocytes, the CFTR inhibitor (R) BPO-27 was used in HC monocytes. This was to establish what role the function as opposed to accumulation of mutated CFTR played in these findings.

I was unable to find any significant difference in mitochondrial damage upon LPS and ATP stimulation when the CFTR inhibitor was used, although this may be because (R) BPO-27 needed a higher concentration or more time to see these cellular effects downstream of CFTR inhibition to take effect. Although a recent publication has shown effective inhibition of CFTR function at similar time and concentrations [433].

However, there were some metabolic differences found between HC monocytes with and without (R) BPO-27, as maximal respiration was found to be downregulated in monocytes with (R) BPO-27 added, this is contrary to what was seen in in CF patient monocytes. It is unclear how inhibition of CFTR would lead to a decrease in maximal respiration. Addition of LPS and ATP lead to an increase in ECAR in HC monocytes without (R) BPO-27 but not in monocytes with (R) BPO-27, again it is unclear why this is the case, but for both of these differences it may just be due to variability between primary cells and use of a low n number; therefore, this would need to be repeated with more samples and with a higher concentration of (R) BPO-27.

As there was no change in mitochondrial damage or metabolism, upon inhibition of the function of WT CFTR, this indicates that the mitochondrial differences seen between HC and CF could largely be due to PTEN. This is because PTEN is a key regulator of metabolism and cell proliferation, but in CF, is unable to bind to ΔF508 CFTR; this implies that the structure of CFTR plays a bigger role in regulation of mitochondrial damage and metabolism than CFTR ion channel function [303].

Chapter 6.0 – Cysteamine and EGCG as a therapy in CF

6.1 Introduction

As autophagy has been reported to be defective in CF [79, 334, 337, 364], several studies have focused on therapies with the potential to recover this mechanism, and therefore, improve the phenotype found in CF ΔF508 homozygous cells [330, 339, 340, 343-346, 453]. The combination therapy of cysteamine and EGCG has been extensively covered in the introduction; in summary, it has been suggested that autophagy is dysregulated due to TG2 being overactive, thereby creating cross-linkages between multiple autophagy proteins, and leading to autophagy not functioning correctly. Cysteamine has been reported to inhibit TG2 and, therefore, recover autophagy function [79, 339, 340], EGCG complements cysteamine by improving the stability of CFTR and, therefore, increasing CFTR cell membrane retention [53, 340], as well as by demethylating the autophagy protein, Atg12 [337]. Cysteamine has also been reported to have mucolytic activity, with disruption of biofilm formation, and helping to kill *P. aeruginosa, B. cepacia*, and antibiotic resistant pathogens [343-346, 453]. EGCG has also been reported to inhibit NF-κB and JAK-STAT activity, via inhibition of CK2 [348-350]. However, a combination of both treatments has been reported to be ineffective in patients with rare CFTR mutations [339].

Despite the development of new CFTR modulator therapies, there is still considerable interest in other therapies for CF; this is mainly due to the high cost of current CFTR modulator therapies, therefore cheaper alternatives may still have a role to play in the treatment of CF. For instance, CFTR modulator therapies have been shown to have an anti-inflammatory effect [63], but little work has been done on investigating whether cysteamine and EGCG also exhibit anti-inflammatory properties in CF. Given how the addition of these CFTR modulators have failed to improve the function of autophagy, as outlined in previous chapters but still exhibit anti-inflammatory properties, I decided to investigate whether the recovery of autophagy would lead to a reduction in the inflammatory signature.

6.2 Results

6.2.1 Inflammation in CF PBMCs with cysteamine and EGCG

To establish the anti-inflammatory properties of cysteamine and EGCG on PBMCs, ELISA assays for TNF, IL-6 and IL-10 were utilised. Cysteamine and EGCG were used, both separately and in combination; it was expected, due to previous studies, that any anti-inflammatory effect the treatment might have would be more potent in combination than when administered separately. The concentrations and times used for cysteamine and EGCG stimulation were acquired from multiple previous studies, using these molecules [339, 340, 343, 346, 453].

Under basal conditions there was no significant differences between HC and CF samples for TNF, IL-6 and IL-10 (Figure 6.1). However, upon LPS stimulation, there was a significant increase in both TNF and IL-6 (Figure 6.1A and B respectively), in comparison to basal conditions for both HC and CF, with a significant difference found between HC and CF with TNF. IL-10 secretion also significantly increased upon LPS stimulation, with the HC samples but not the CF samples, and there was a significant difference between HC and CF upon LPS stimulation (Figure 6.1C). The addition of cysteamine and EGCG, on their own, elicited no response for any of the cytokines (Figure 6.1A-C). The addition of cysteamine had no effect on cytokine secretion, on LPS stimulation. However, the addition of EGCG significantly reduced the secretion of TNF in CF PBMCs, on LPS stimulation, and significantly reduced IL-6 in both HC and CF PBMCs (figure 6.1B). IL-10 secretion from HC PBMCs showed a significant reduction, upon EGCG and LPS stimulation compared to LPS (Figure 6.1C). Cysteamine and EGCG in combination didn't show any increase in cytokine secretion. Cysteamine and EGCG, in association with LPS stimulation, showed a significant reduction in cytokine secretion compared to LPS (figures 6.1A-C).

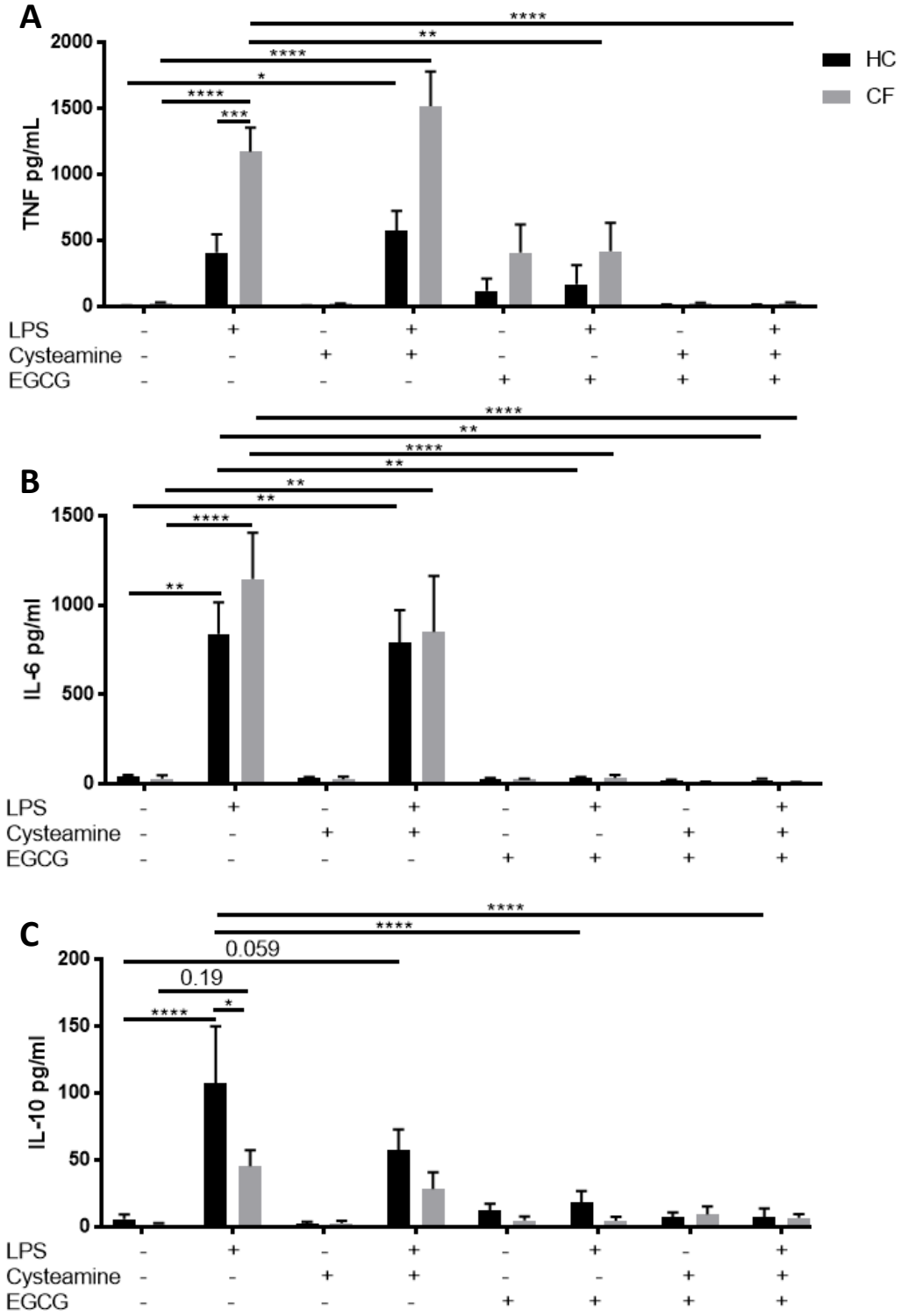


Figure 6.1 – TNF, IL-6 and IL-10 secretion in PBMCs with cysteamine and EGCG. ELISA assays were used to detect (A) TNF, (B) IL-6 and (C) IL-10 secretion in both HC and CF PBMC supernatants. Stimulations used are 4 hours stimulation with LPS (10 ng), 24 hours cysteamine (250 µM) and 24 hours EGCG (100 µM). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=8 for TNF, n=7 for IL-6 and n=5 for IL-10, where n is the number of different HC or CF samples.

6.2.2 Annexin V live/dead assay with cysteamine and EGCG in HC PBMCs

As EGCG was able to dramatically reduce the secretion of TNF, IL-6 and IL-10 , for both HC and CF PBMCs, I decided to investigate the toxicity of EGCG at different concentrations (25, 50, and 100 µM). I also ran a sample with different concentrations of cysteamine (50, 100 and 250 µM) as a control. To achieve this, I utilised flow cytometry and the Annexin V live/dead assay (Sigma-Aldrich).

Representative MFIs are shown in Figures 6.2C, with no propidium iodide in Figure 6.2D and no annexin V in Figure 6.2E. Due to the low number of replicates, statistical analyses could not be carried out on Figure 6.2A and 6.2B.

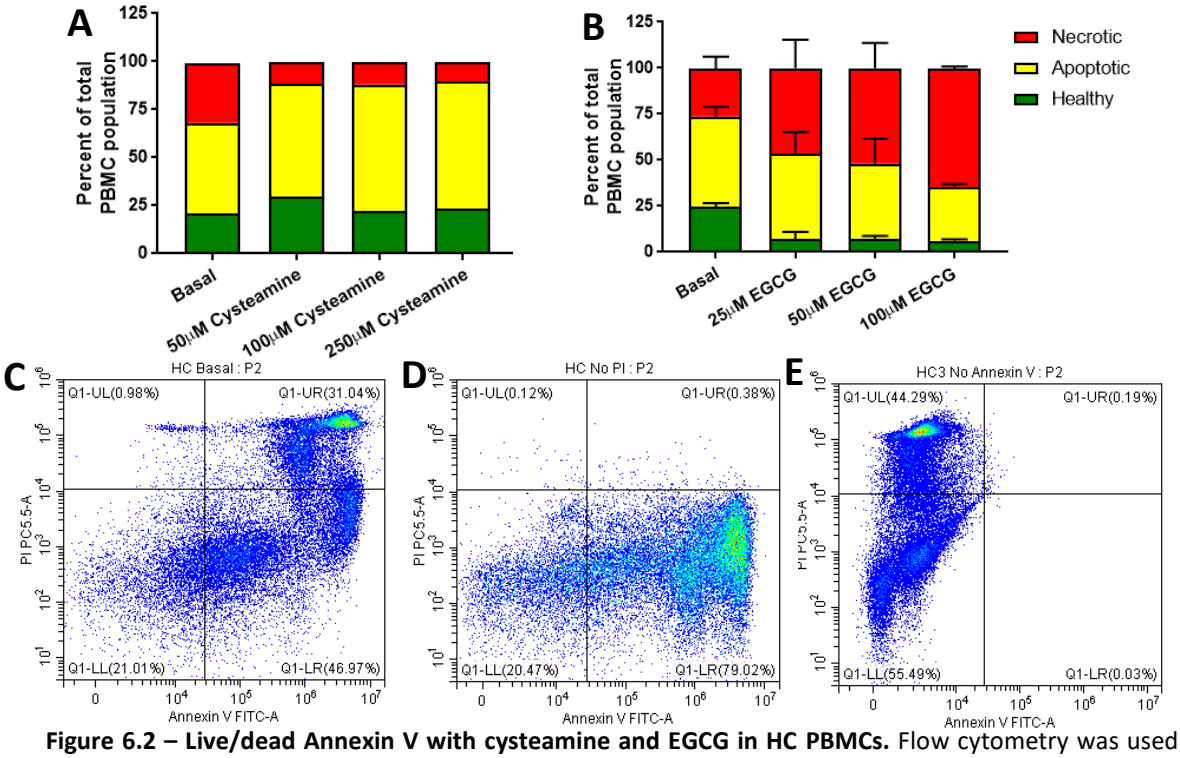


Figure 6.2 – Live/dead Annexin V with cysteamine and EGCG in HC PBMCs. Flow cytometry was used to measure proportion of HC PBMCs that were either in a healthy, apoptotic or necrotic state. Gating strategy shown in Appendix Figure 5. Cells were stimulated with (A) cysteamine at 50 µM, 100 µM and 250 µM, (B) EGCG was used at 25 µM, 50 µM and 100 µM. (C) Representative MFI, (D) no PI, (E) no annexin V. All data are presented as mean ± SEM. n=1 for cysteamine and n=3 for EGCG, where n is the number of different HC or CF samples.

6.2.3 Annexin V live/dead assay with fresh EGCG in HC PBMCs

As EGCG was found to result in a significant increase in necrosis, I decided to try fresh EGCG, as opposed to the aliquoted EGCG that had been frozen and then thawed for use. Annexin V live/dead assay was utilised again, with flow cytometry using a broader range of EGCG concentrations with HC PBMCs; representative MFI are shown in Figure 6.3A. Due to the low number of replicates, statistical analyses could not be carried out on Figure 6.3B.

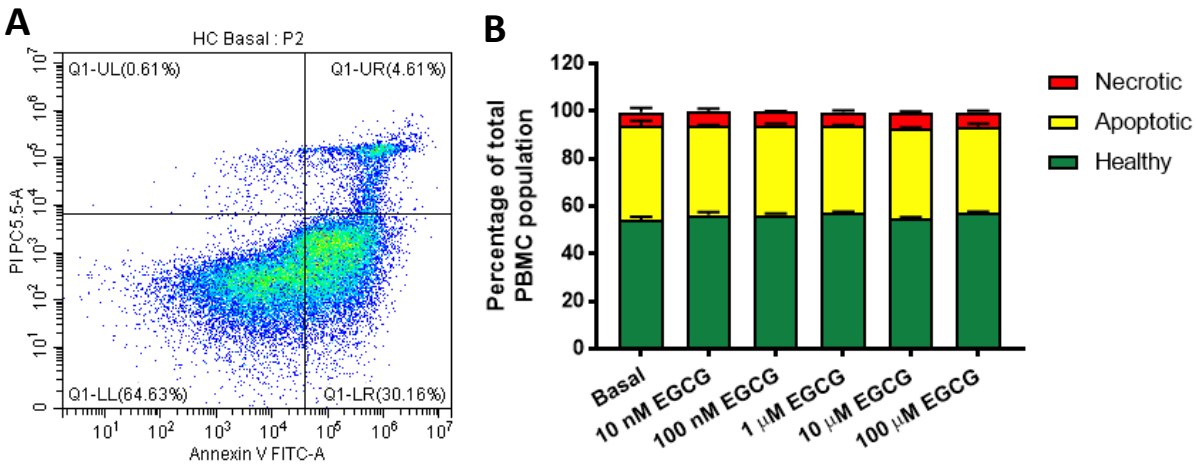


Figure 6.3 – Live/dead Annexin V with fresh EGCG in HC PBMCs. Flow cytometry was used to measure proportion of HC PBMCs that were either in a healthy, apoptotic or necrotic state. Gating strategy shown in Appendix Figure 5. Cells were stimulated with (A) cysteamine at 50 µM, 100 µM and 250 µM, (B) EGCG was used at 25 µM, 50 µM and 100 µM. (C) Representative MFI, (D) no PI, (E) no annexin V. All data are presented as mean ± SEM. n=3, where n is the number of different HC or CF samples.

6.2.4 Inflammation in HC PBMCs after fresh EGCG

Instead of using frozen and then thawed EGCG, fresh EGCG was used to see if it had any effect on inflammation in HC PBMCs. To achieve this ELISA assays for both TNF and IL-6 were performed, using a range of fresh EGCG concentrations (Figure 6.4A and B respectively). Upon addition of LPS there was a significant increase in secretion of both TNF and IL-6, compared to basal conditions (Figure 6.4A and B respectively). With increasing concentrations of EGCG, from 10 µM to 200 µM, there was no reduction in the secretion of TNF or IL-6 compared to LPS on its own.

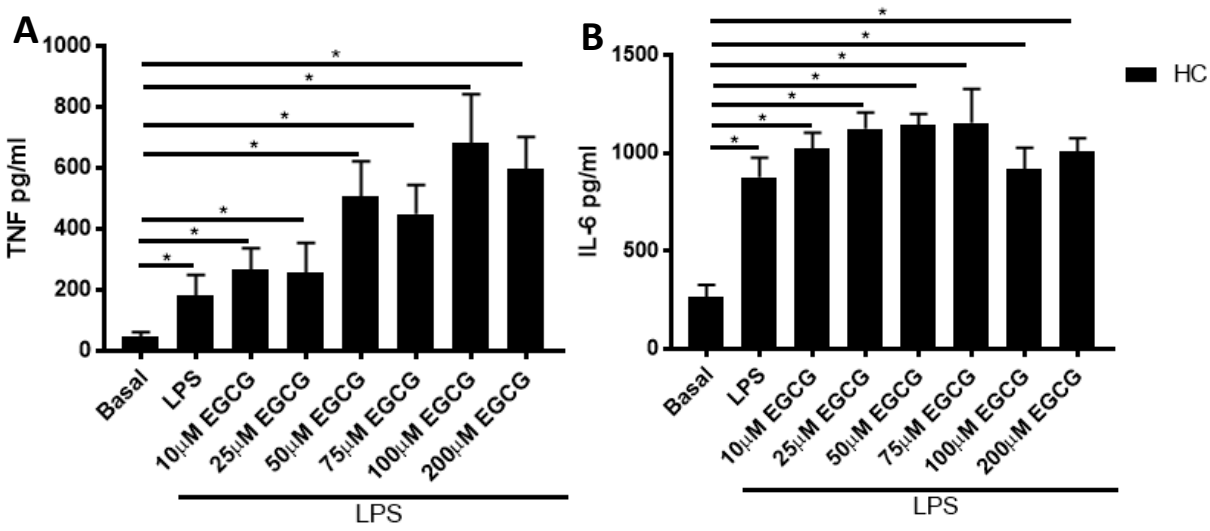


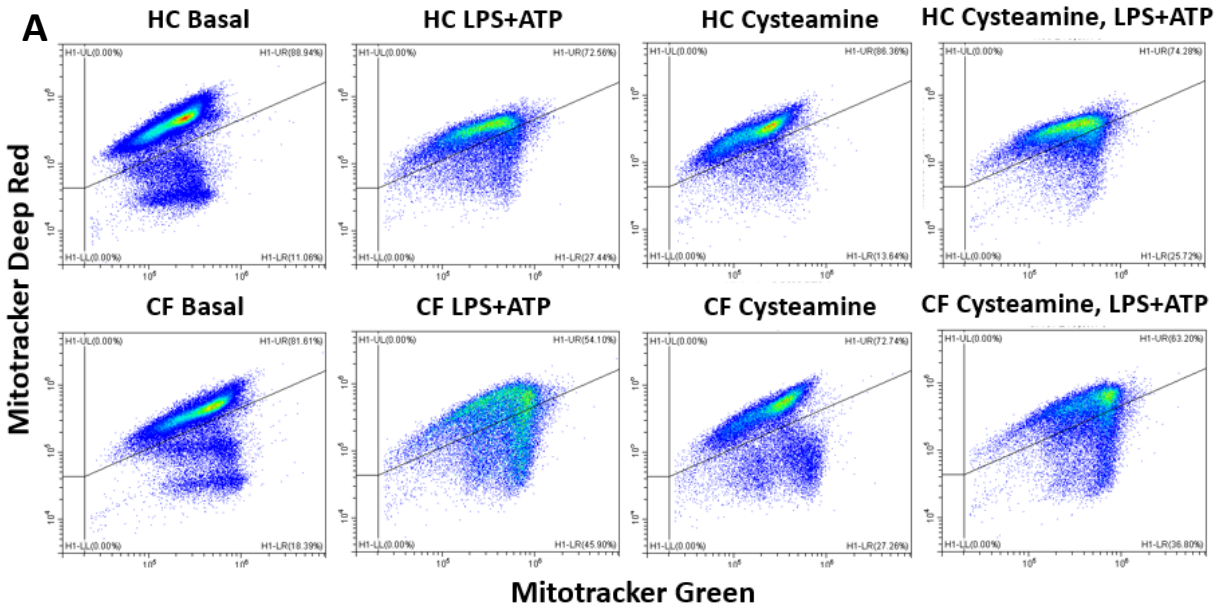
Figure 6.4 – TNF and IL-6 secretion in HC PBMCs with fresh EGCG. ELISA assays were used to detect (A) TNF and (B) IL-6 and secretion in HC PBMC supernatants. Stimulations used are 4 hours with LPS (10 ng) and 24 hours EGCG (10, 25, 50, 75, 100 and 200 µM). All data are presented as mean ± SEM. Statistical significance was calculated using a two-way ANOVA multiple comparisons statistical test with Tukey correction (p values *=≤ 0.05, **=≤ 0.01, ***=≤ 0.001 and ****= ≤ 0.0001). n=4, where n is the number of different HC or CF samples.

6.2.5 Recovery of autophagy and mitochondrial damage

As cysteamine has been shown to recover autophagy in several studies, and in the previous chapter I have shown mitochondrial damage which may be linked to dysregulated autophagy, I decided to investigate whether cysteamine was able to improve the state of mitochondria in CF monocytes, by utilising the MTDR and MTG flow cytometry assay from the previous chapter. LPS and

ATP were used to induce mitochondrial damage, and cysteamine was used to see if it helped to prevent mitochondrial damage.

Representative MFIs are shown in Figure 6.5A. Due to low number of CF samples, I was unable to perform stats on this experiment.



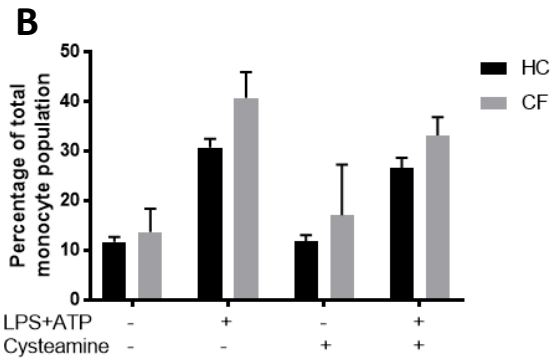


Figure 6.5 – Mitochondrial damage in HC monocytes with cysteamine. Flow cytometry was used to measure mitochondrial damage in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5mM) for the last 0.5 hours. Cysteamine was used for 24 hours at 250 µM. Respiring mitochondria were stained with Mitotracker Deep Red (MTDR) and all mitochondria were stained with Mitotracker Green (MTG), both dyes were acquired from Invitrogen. Gating strategy shown in Appendix Figure 3. Representative mean fluorescence intensity (MFI) (A) and percentage of damaged/depolarised mitochondria quantified (B). All data are presented as mean ± SEM. n=4 for HC and n=2 for CF, where n is the number of different HC or CF samples.

6.2.6 Role of NLRP3 inflammasome in mitochondrial damage

As LPS and ATP are classic NLRP3 inflammasome inducers, and NLRP3 inflammasome activation has been reported to prevent clearance of damaged mitochondria, as a positive feedback loop to further activate the inflammasome, I decided to investigate how much of a role the overactivation of the NLRP3 inflammasome in CF plays in preventing the clearance of damaged mitochondria in CF.

The caspase-1 inhibitor, ac-yvad-cmk, was utilised with the MTDR and MTG flow cytometry assay, and representative MFIs are shown in Figure 6.6A. This experiment was run at the same time as Figure 6.5, so the results shown for basal and LPS with ATP are the same in Figure 6.5 as Figure 6.6. Again, due to the low number of replicates, statistical analyses could not be performed on Figure 6.6B.

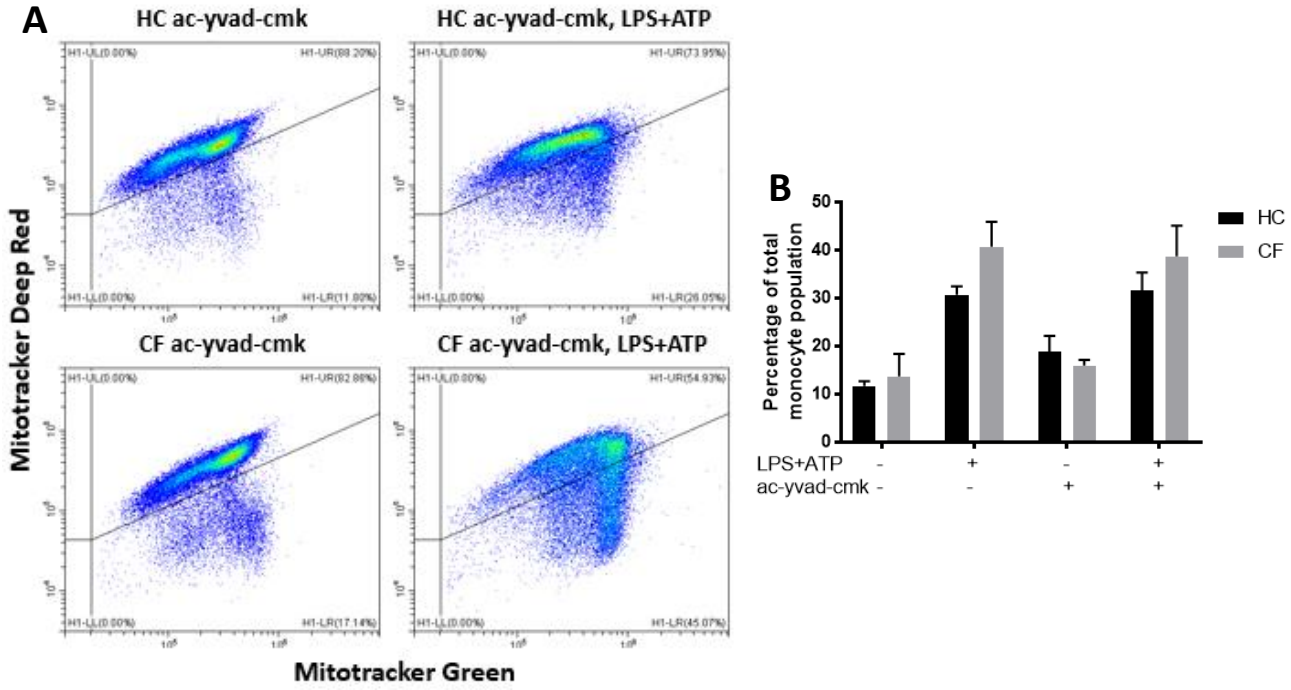


Figure 6.6 – Mitochondrial damage in HC monocytes with caspase-1 inhibitor. Flow cytometry was used to measure mitochondrial damage in both HC and CF monocytes under basal conditions and after 4 hours stimulation with LPS (10 ng) and the addition of ATP (5mM) for the last 0.5 hours. Caspase-1 inhibitor, ac-yvad-cmk was used at 2µg/ml for 1 hour. Respiring mitochondria were stained with Mitotracker Deep Red (MTDR) and all mitochondria were stained with Mitotracker Green (MTG), both dyes were acquired from Invitrogen. Gating strategy shown in Appendix Figure 3. Representative mean fluorescence intensity (MFI) (A) and percentage of damaged/depolarised mitochondria quantified (B). All data are presented as mean ± SEM. n=4 for HC and n=2 for CF, where n is the number of different HC or CF samples.

6.3 Discussion

6.3.1 Impact of cysteamine and EGCG on inflammation

As inflammation plays such a large role in the pathogenesis of CF, development of new therapies which help to reduce inflammation is vital for the long-term wellbeing of patients with CF. This chapter reports that the combination of cysteamine and EGCG failed to produce beneficial anti-inflammatory effects *in vitro*. The anti-inflammatory impact of cysteamine and EGCG in Figure 6.1 may be due to necrosis of the cells, which might be largely due to EGCG being used after it was frozen and then thawed for use. Fresh EGCG was then used to investigate if fresh EGCG induces necrosis, but no conclusion could be drawn due to the low number of replicates. It is unclear why the freeze-thaw process might have such a profound impact on the effect EGCG has on the cells. However, as far back as 2004, EGCG has been reported to induce protein expression and activate caspases 3, 8 and 9, driving the cells towards apoptosis, and subsequently, it has been of interest as a preventative treatment against cancer [348]. Other reports have shown EGCG to induce apoptosis and depolarise mitochondria in the hepatocellular carcinoma cancer cell line, HCCLM6, but not in non-cancerous liver cells [454], as well as its ability to promote apoptosis in a number of cancer cell lines, including the human breast cancer cell line MCF-7 [455], as well as human adrenal cancer NCI-H295 cells [456], and in HepG2 cancer cells [457].

Despite this, several other studies have also found EGCG to possess various anti-inflammatory and anti-oxidant properties [348, 458-461]; however, due to its ability to promote apoptosis, it may not be of particular use in autoinflammatory diseases, such as CF, apart from in certain specific contexts where apoptosis would be beneficial, such as with neutrophils in the lungs of patients with CF. More recently, EGCG has been shown to inhibit both canonical and non-canonical inflammasome activation in LPS and amyloid-β induced microglial cells *in vitro* and *in vivo* in a mouse model [462]. In addition, EGCG has been reported to inhibit intracellular ROS production and suppress mtDNA

synthesis to prevent NLRP3 inflammasome activation in mouse macrophages, thereby preventing the progression of acute gout [463].

The combination of cysteamine and EGCG has been reported to improve the stability and function of the CFTR protein [339, 340], as with CFTR modulators. As it has recently been shown that these CFTR modulators have anti-inflammatory effects [63], it is possible that, as well as the anti-inflammatory effects helping the recovery of autophagy [339, 340], the improved function of CFTR protein with cysteamine and EGCG may be partly responsible for the inhibition of inflammation. However, a recent report, by Armirotti *et al* 2019, indicated that cysteamine does not rescue CFTR function, and furthermore, that study also purported to show that cysteamine and EGCG, when used together, have deleterious effects upon bronchial epithelia [464].

Other potential anti-inflammatory and autophagy modulating therapies have been investigated, such as the innate defence regulator (IDR)-1018, which is an anti-inflammatory peptide found to induce autophagy, via modulation of AMPK. In flagellin-stimulated CF cells, IDR-1018 was shown to induce the clearance of LC3B and autophagosomes, leading to a reduction in inflammatory markers [465], thus indicating the potential benefits of recovering autophagy on the inflammatory phenotype in CF. The polypeptide, thymosin α-1 (Tα-1), has also been proposed, by Romani *et al* as a potential autophagy and CFTR corrective therapy in CF, in 2017 [466]; however, these results were called into question when other laboratories were unable to replicate the results [467, 468]. This development has lead to Romani *et al* publishing a response questioning some of the methodology of these studies [469], which was then answered with a further report, again showing no improvement of CFTR function with Tα-1, and ending with the recommendation that both Tα-1 and cysteamine not be used in future clinical trials, due to demonstrable lack of efficacy [464].

6.3.2 Impact of cysteamine and ac-yvad-cmk on mitochondrial damage

The variability in n number, between HC and CF used for these experiments, was due to the patient cohort for this study starting to run out. This is because of the introduction of CFTR modulator

therapies, as standard care for all patients with CF, as opposed to just patients who are prescribed CFTR modulators on compassionate grounds. As a result of this I was unable to obtain any further samples which are comparable to previous samples collected.

Due to the low n number for CF samples, nothing conclusive can be drawn from the cysteamine and caspase-1 inhibitor, ac-yvad-cmk, mitochondrial damage assays. However, a tentative hypothesis can be considered for these assays. The addition of cysteamine to the mitochondrial damage assay appears to partially lower the quantity of damaged mitochondria in CF monocytes, closer to that of HC. Based on published literature on the use of cysteamine in CF, this could be due to cysteamine's ability to aid in the recovery of autophagy, and, therefore, to improve clearance of damaged mitochondria [339, 340]. Inhibition of caspase-1 would be expected to lead to an improvement in mitochondrial quality, due to caspase-1's ability to prevent clearance of damaged mitochondria.

Chapter 7.0 – Discussion

The aim of this study was to further clarify the state of autophagy in innate immune cells, containing CF mutations, and to investigate whether there were specific cellular abnormalities that could be linked to any dysregulation of autophagy found. The project was divided up into four main objectives: (1) Clarify whether HBECs with CF mutations show any dysregulation of autophagy. (2) Investigate the impact homozygous ΔF508 CFTR mutations have on autophagy in patients' primary cells. (3) Establish whether mitochondria are abnormal in CF, as a possible result of dysregulated autophagy. (4) To investigate the impact small molecule autophagy-augmenting therapies would have on inflammation and mitochondria in CF.

7.1 Autophagy is dysregulated in CF cells

This study has shown that autophagy is dysregulated in CF; however, there were some interesting differences found between dysregulated autophagy in CF HBECs and primary cells from CF patients, bearing the same mutations. Following up on previous published studies on autophagy in CF, which were focused on sub-optimal HBEC cell line comparisons between a CF cell line (IB3-1) and a "corrected" IB3-1 cell line (C38), I decided to try and produce an improved comparison study between an actual WT HBEC cell line and several other CF HBEC cell lines. I found that, in the cell lines, there was an increased autophagic flux in the CuFi-1 cell line but not in the IB3-1 cell line, relative to WT, with a normal turnover of p62. However, the primary patients' monocytes and M1 macrophages had a significantly increased level of p62 but no change in autophagic flux. CFTR modulators failed to alter autophagic flux for both cell lines and primary cells. The reason why CF HBECs have a different kind of dysregulation of autophagy to CF primary cells is unclear; however, a few reasons may be suggested, such as the HBECs have been genetically modified, which might have altered the autophagic mechanism in some way. Also, the HBEC cell lines are from different patients and, therefore, will present with some degree of variability anyway, not just based on the different mutations. Another reason could be due to the fact that they are different cell types and, as the CFTR is expressed to

different degrees in each cell type, perhaps the increased level of expression of mutated protein in epithelial cells alters the autophagy mechanism in a different way to the lower level of expression found in monocytes and M1 macrophages, and this could be due to the different levels of protein aggregation found across these cell types.

Several studies investigating CFTR modulators that also augment autophagy have focused on the E3 ubiquitin ligase, RNF5/RMA1, which is involved in the early stages of CFTR biosynthesis and promotes its degradation [470]. It was found, via screening of an siRNA library for known CFTR interacting proteins, that inhibition of RNF5 results in a significant rescue of ΔF508 CFTR. The same study also showed that RNF5 inhibition resulted in improved intestinal absorption in mice and led to an improvement in CFTR function in intestinal epithelial cells [471]. Furthermore, a recent study identified inh-02 as a small molecule RNF5 inhibitor that reduces ubiquitylation of ΔF508 CFTR, rescues ΔF508 CFTR function and augments autophagy [472]. This augmentation of autophagy was linked to regulation of Atg4B and LC3 by RNF5, modulating the basal level of autophagy [473].

Lysosomes also exhibited differences between CF HBECs and CF primary monocytes and M1 macrophages, as M1 macrophages were found to have a raised concentration of LAMP1 relative to HC. Raised expression levels of LAMP1 have been associated with the progression of a number of diseases, such as Alzheimer's disease [474] and several types of cancer [475-477], raising the possibility that patients with CF are also at a higher risk of developing other diseases. It is unclear why these differences between these cell types exist; however, it could be partially due to the same reasons mentioned above. The work presented here did not investigate whether CFTR function plays a role in the pH calibration of lysosomes but did fail to find any dysregulation of either the quantity or function of lysosomes. Whether lysosomes in CF have an altered pH and whether this is linked to the function of the CFTR protein remains controversial, with studies purporting to show that either the ion channel does have an impact on lysosomal function [111, 112, 358, 360] or does not [113, 114, 359]. Given that the CFTR plays an important role in modulating the extracellular pH in the ASL, it would be surprising if the CFTR did not also contribute to regulating the pH of lysosomes in the cell.

The latest study, in 2012, reported that although the mutated CFTR does not alter baseline lysosomal pH, the CFTR still plays a vital role in the reacidification of alkalised lysosomes, both in epithelial cells and in isolated lysosomes [360].

There are several similarities between CF and neurodegenerative diseases as protein aggregation disorders; for example, dysregulated autophagy and heightened inflammation are prevalent pathologies found in both disorders. It is well established that low grade systemic inflammation is found in neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, with an inflammatory phenotype of increased IL-1β, TNF and IL-6 markers found in cerebral spinal fluid and serum [478-480]. Neuroinflammation in neurodegenerative disorders in recent years has been investigated, with the aim of establishing whether this neuroinflammation is just a symptom of these disorders or is also a contributing factor to the pathology of these patients. There have been several studies recently which have focused on inhibiting inflammation in these disorders and reporting a reduction in the rate of cognitive decline [481, 482]. Based on the fact that CF patients have low grade systemic inflammation [483] and the CFTR is found throughout the brain [19, 484-488], there is the potential, that as survival increases in people with CF, a similar rate of decline in cognition and potentially dementia could occur, and be comparable to these neurodegenerative disorders. The introduction of CFTR modulators, as standard care for patients with CF in the UK was a significant milestone for CF patients' treatment; however, with the increased life span of these patients there may be new challenges to overcome. The introduction of anti-inflammatory therapies in CF and the finding that CFTR modulators have anti-inflammatory properties themselves [63] could potentially act as a protective strategy in preventing any cognitive decline that might occur.

This thesis focused on macroautophagy, the other forms of autophagy, microautophagy and CMA have not been investigated so far. As the main mechanism of autophagy is dysregulated in CF, it is likely that the other forms of autophagy are defective in some way too. For example, the chaperone protein, Hsp70, is essential for CMA, as it binds to misfolded proteins and targets them for degradation

via direct fusion with lysosomes via LAMP-2A [188]. As Hsp70 has been found to associate with ΔF508 CFTR due to exposed hydrophobic residues and ΔF508 CFTR has been found to aggregate in the cell [47, 48], it is possible that Hsp70 binds to this aggregated CFTR protein but is unable to clear it. This would mean this chaperone protein would be unable to carry out its usual function as it is retained in the aggresomes.

Autophagy plays a vital role in multiple cellular processes, with autophagy being dysregulated in CF, there may be abnormalities in these other cellular processes not covered in this thesis. Regarding the immune system, autophagy is key for its correct function, and although this thesis focuses on innate immunity in CF, autophagy is also important for the adaptive immune system. Autophagy regulates lymphocyte cell differentiation and survival, as well as antigen processing and presentation; and so, autophagy's dysregulation is likely to have an impact on these processes. Autophagy's dysregulation may play a role in the adaptive immune dysfunction in CF with hyperinflammatory CD3+ lymphocytes, disrupted Th17 and Th2 cell responses, as well as downregulation in Treg population and upregulation in invariant NK cells [439, 489, 490]. The impact correction of autophagy would have on these cellular defects in CF would be highly interesting, especially in clearance of bacterial infections.

7.2 Mitochondria and metabolism are abnormal in CF monocytes

Correctly functioning mitochondria are profoundly important in maintaining proper cell function, with abnormal/damaged mitochondria being linked to a plethora of inflammatory diseases [158, 159, 164, 170, 180]. As previous studies have shown that deficient autophagy results in an accumulation of damaged mitochondria and heightened NLRP3 inflammasome activation [231], I decided to investigate whether this is the case in CF. Furthermore, cell lines treated with ethidium bromide and lacking mtDNA, exhibit impaired caspase-1 activation and, therefore, reduced IL-1β and IL-18 production [231, 491], underlining the link between autophagy dysregulation, mitochondrial damage and NLRP3 inflammasome activation.

This study has demonstrated that there were significant differences in both mitochondrial integrity and function in CF monocytes relative to HC, and also, that mitochondria in CF monocytes were more depolarised upon stimulation, producing greater amounts of mROS with significantly increased metabolic rates relative to HC. Although I was unable to fully link these results to dysregulated autophagy, it is likely that the recovery of autophagy in CF would lead to improved mitochondrial integrity, and a partial reduction in the heightened metabolic state. However, further work will be required to establish a definitive link between dysregulated autophagy and the accumulation of damaged mitochondria and altered metabolism. Metabolism has been shown to be a crucial determinant in proper cell differentiation, and the finding that metabolism is abnormal in CF may partly explain why certain cell populations are abnormal in CF [28]. A recent study has established that, in macrophages, the NLRP3 inflammasome helps to modulate glycolysis in an IL-1β dependent manner. IL-1β was found to upregulate the glycolytic enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), an essential driver of glycolysis via its modulation of a rate limiting step in glycolysis [492]. It will be most interesting to establish what effect(s) the introduction of CFTR modulator therapies have on metabolism in CF.

As a result of pancreatic malabsorption and increased cellular metabolism, driven in part by both infection and inflammation, patients with CF require a high calorie and high fat diet (HFD). However, there is evidence that HFDs may contribute to systemic inflammation and lung fibrosis [493-495]. Recently, a HFD in mice has been linked to the downregulation of an autophagy and Nrf2 regulating protein, sestrin2, leading to a reduction of the Nrf2 oxidative stress response [496]. Sestrin2, once activated, has been found to induce autophagy via activation of AMPK, leading to inhibition of mTOR and subsequent stimulation of autophagy. This induction of autophagy has been linked to activation of the Nrf2 pathway, via autophagic degradation of KEAP1, leading to Nrf2 release and increased nuclear translocation with consequent increased anti-oxidant gene expression [497]. The mice with a HFD also showed reduced insulin sensitivity, which, along with the high glucose diet and CFTR-driven pancreatic dysfunction [498], may help explain why diabetes is so prevalent in people

with CF [496]. Furthermore, an accumulation of damaged mitochondria within β-cells has been associated with the development of diabetes [499]. Another study, also in mice, reported that sestrin2, via its induction of autophagy, plays a protective role against obesity-induced insulin resistance and helped to prevent diabetes [500]. These factors may contribute to the altered metabolic state and damaged mitochondria seen in CF.

Clearance of damaged mitochondria has proven to be a double edged sword, with excessive clearance resulting in inhibited apoptosis, prolonged cell survival and development of fibrosis [501], whereas, dysregulated clearance results in inflammation and fibrosis [251, 502]. Recently, the mitochondrial protein, PGAM5, has been found to be a key driver of mitochondrial dysfunction in idiopathic pulmonary fibrosis (IPF), with PGAM5$^{-/-}$ mice showing reduced lung fibrosis relative to controls [416]. PGAM5 was reported to maintain mitochondrial integrity of mitochondrial structure and function; however, blockage of mitochondrial clearance improved mitochondrial homeostasis. This indicates that PGAM5 can improve mitochondrial function but also induce mitochondrial damage depending on the context [416].

Mitochondria also undergo fusion and fission to maintain a healthy population of mitochondria. Under stressed conditions the balance between fusion and fission is tipped towards fission to ensure swift amputation and removal of damaged sections of mitochondria via mitophagy. As mitochondrial damage has been shown to be present in CF monocytes, it is likely that mitochondria will be undergoing an increased rate of mitochondrial fission. Asymmetric mitochondrial fission results in accumulation of mitochondria with varying ΔΨm, which would then, under normal conditions, be cleared via autophagy [499, 503]; however, due to dysregulated autophagy, this might not be the case in CF. This could mean that due to the oxidative stress found in CF cells, an upregulation in mitochondrial fission might be partly responsible for the increase in damaged mitochondria found in CF monocytes. Moreover, inhibition of either mitochondrial fission proteins Drp1 or OPA1 is enough to result in an accumulation of damaged mitochondria [499].

Transforming growth factor (TGF)β is a cytokine that has been shown to be a master regulator of pulmonary health and disease [504]. Polymorphisms of TGFβ, C-509T and T29C, have been found to be linked to a more severe form of CF lung disease, with increased expression of TGFβ [505, 506]. Increased expression of TGFβ has been found to result in mitochondrial depolarisation, increased mROS and PINK1 production and increased lung fibrosis [507]. Therefore, it is possible that raised TGFβ found in CF, could be linked to the abnormal mitochondria found in CF monocytes.

A dysregulated UPR has been linked to a heightened inflammatory and metabolic state in CF [103], so with the obvious links between the ER and mitochondrial function, it is likely that a dysregulated UPR would also contribute to the mitochondrial dysfunction seen in CF [323, 324, 508]. In addition, the antioxidant MitoQ, has been shown to protect against acute lung injury in rats, highlighting the link between the mitochondrial dysfunction and lung injury [509]. Mitochondrial and pulmonary dysfunction are well-known characteristics of the protein aggregation disorder, Parkinson's disease [510, 511], further exemplifying the similarities between CF and other protein aggregation disorders. From these results it is clear that no one feature is responsible for the mitochondrial dysregulation seen in CF, but several overlapping causes are present, feeding into each other. Further research is therefore required to elucidate how much of a role CFTR function plays in mitochondrial dysregulation.

7.3 Cysteamine and EGCG as therapy in CF

Despite the interest in cysteamine and EGCG in the past decade, no clear picture has emerged as to whether cysteamine and EGCG improve CFTR processing and membrane retention, with studies claiming to show both an improvement [339, 340] and lack of improvement [464]. This study aimed to clarify whether cysteamine and EGCG, either in combination or independently, had any anti-inflammatory properties that would be of benefit in CF, as well as whether cysteamine could improve mitochondrial integrity. These results demonstrate that neither molecule had an anti-inflammatory impact, but cysteamine might improve mitochondrial integrity; however, this result is extremely

tentative due to the low n numbers used. The results also fail to show any role played by the NLRP3 inflammasome in mitochondrial damage.

Trying to reduce inflammation in CF has been suggested to be highly important, as drugs that target inflammation have been shown to reduce the rate of decline in lung function as well as improving survival [512, 513]. New anti-inflammatory therapies are being investigated for CF, such as the potential use of the NLRP3 inflammasome inhibitor, MCC950 [185], as well as the inhibition of ENaC also decreasing NLRP3 inflammasome activation [28].

The augmentation of autophagy in general for various diseases has been considered already, with research on autophagy stimulation in COPD [514], neurodegenerative disease [515], cardiovascular disease [516], and Crohn's disease [517]. Targeting dysfunctional mitochondria and improving mitophagy have been considered as potential therapeutic targets for some time, with potential benefits in several diseases, such as neurodegeneration [518], cancer [519] and cardiovascular disease [520]. Mitophagy-inducing therapies that don't damage the mitochondria would be of significant interest in CF, as they could establish whether there is any therapeutic benefit to clearing damaged mitochondria, with the aim of reducing inflammation and decreasing the raised metabolic state to normal levels.

7.4 CF as an autoinflammatory disorder

The definition of autoinflammatory disease is the excessive activation of the innate immune system in the absence of infection, leading to increased production of inflammatory cytokines; an adaptive immune response such as B cells, T cells and autoantibodies is absent [142, 152]. With the finding that dysregulated autophagy, abnormal mitochondria and a hyperinflammatory response are present in CF and that these are all prominent characteristics of autoinflammatory diseases, CF may also be considered an autoinflammatory disease.

CF has been described as an autoinflammatory disease previously [142], with inflammation in the lungs [83], dysregulated UPR-driven inflammation [103], excessive neutrophil infiltration, and an

overactive NLRP3 inflammasome [28, 185]. The recent finding that correction of the CFTR mutation leads to a significant reduction in inflammatory markers adds weight to this argument, as it indicates that the physiological drive towards inflammation is primarily due to CFTR dysfunction [63].

7.5 Concluding remarks

Based on the current literature, and the results shown in this study, it can be concluded that autophagy is dysregulated in CF, and that mitochondrial quality and function are also abnormal with many similar characteristics in CF to that of autoinflammatory diseases. With the recent discovery of NLRP3-driven inflammation in CF [28, 185], and the findings that mitochondria are abnormal and producing NLRP3 inflammasome activating signals, it will be of interest to establish whether recovering autophagy would hold any benefit for patients with CF in future studies.

7.6 Study limitations

There are several noteworthy limitations in this study; firstly, due to the low number of replicates, two of the statistical tests performed were not appropriate. Both the two-way ANOVA test and multiple t-tests are parametric tests assuming a normal distribution, as the number of replicates were low for some of the figures, it was inappropriate to use a parametric test, as you can't assume a normal distribution. This led to low P values for some of the figures where a non-parametric test would have found a much higher P value. Also, as stated in the statistical methods section, no test was carried out to check if samples represented a normal distribution; and therefore, I should not have used parametric statistical tests. More appropriate statistical tests would have been a non-parametric Friedman's two-way ANOVA instead of the two-way ANOVA used, and a non-parametric Mann-Whitney instead of multiple t-tests wherever they were used.

Another limitation to consider is the Seahorse assays baseline reading. The Seahorse assay had multiple HC samples each time the assay was performed, each time the assay was run, every sample was made relative to one of the HC baseline samples. This was to try and standardise between assay runs to try and reduce variability due to human error; however, to get a more accurate baseline

reading, the average of all HC baseline samples should have been used, and then all samples made relative to that, this would have provided a more accurate baseline reading.

In addition, the cell lines are from different individuals, and although the cells underwent the same immortalisation process, there may be different reactions to the immortalisation process, based on different genotypes in the range of individuals. Furthermore, both the Beas-2b and IB3-1 cell lines were grown in the same LHC basal media, whilst the CuFi-1 and CuFi-4 cell lines were cultured in LHC-9 media. Future work would optimally use the WT control cell line, NuLi-1 (ATCC® CRL-4011™), which is grown in the same LHC-9 media as the CuFi-1 and CuFi-4 cell lines. Ideally, primary epithelial cells would be acquired to clarify the state of autophagy in HBECs. Other limitations involve the patient cohort used, which was selected on the basis of CF patients' clinical stability, genotype (ΔF508 homozygous) and age (18 years old and older). The variety of medications the patients were taking was not considered, other than CFTR modulators used, as there is a great range of medications and a large sample size was required. Moreover, M1 macrophages in this study were monocyte-derived macrophages that were grown *in vitro* with GM-CSF; although this is a well-established protocol, there are limitations as to how much this can be considered comparable to *in vivo.* Nearing the end of the study, the patient cohort was beginning to be transferred to CFTR modulator therapies, as the new standard of care; this means that it was not possible to carry out a number of planned experiments near the end of the study. Unfortunately, due to the COVID-19 pandemic, several experiments also had to be cancelled.

7.7 Future Directions

To make the cell lines more comparable, it would be useful to utilise CRISPR/Cas9 to either insert the faulty *CFTR* gene, *ΔF508*, into the Beas-2B cell line or knockout the *CFTR* gene, this would allow for a direct comparison between a WT HBEC cell line, a homozygous ΔF508 cell line and a KO CFTR cell line. Another line of investigation would be to acquire the NuLi-1 cell line (ATCC® CRL-4011™),

as it is a cell line specifically developed to be the WT HBEC for both the CuFi-1 and CuFi-4 cell lines and is also grown using the same LHC-9 media.

I plan to use the RNA-seq next generation sequencing service, provided by Novogene, to sequence the mRNA of 5HC and 5CF monocyte samples, under basal conditions. This will provide me with a huge amount of data to compare the expression of genes between HC and CF monocytes, I intend to use this data to compare both autophagy, mitochondrial and NLRP3 inflammasome related genes to further characterise their state in CF.

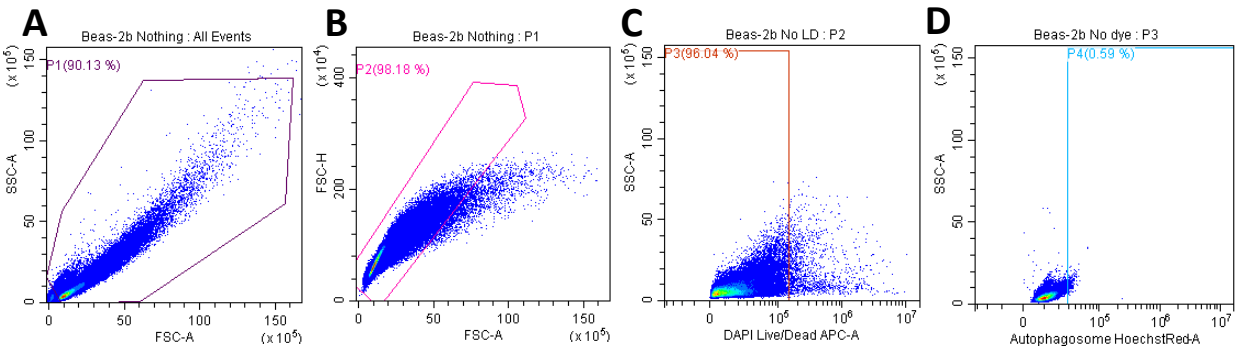
Another mechanism that plays an essential role in maintaining cellular homeostasis through protein degradation and clearance involves the proteasome pathway; in this study I have established that autophagy was dysfunctional, but it would also be useful to clarify the state of the proteasome pathway. Another important experiment would be to solidify the link between dysregulated autophagy and mitochondrial abnormalities. In this regard, it would be ideal to isolate mitochondria from monocytes with homozygous ΔF508 CFTR mutations, and perform western blots to establish whether autophagy proteins are correctly associating with the mitochondria in CF. I predict that there would be an accumulation of p62 and LC3BII on mitochondrial membranes in CF, and that these p62-bound mitochondria will not be cleared from the cell correctly, due to dysregulated autophagy.

With the introduction of CFTR modulator therapies, as standard care, it will be of interest to track the changes in mitochondrial function and p62 expression, as this protein binds to ubiquitinated proteins and organelles to target them for degradation. The overall concentration of ubiquitin in the cell could be quantified as well. I hypothesise that there will be a reduction in metabolic rate as well as a decrease in p62 and ubiquitin concentration in CF cells. The link between Ca^{2+} and mitochondrial integrity and function in CF should be further investigated, as the recent findings linking Na^{+} and K^{+} ionic imbalance to NLRP3 inflammasome in CF indicate that other ionic imbalances may be present and important to the excessive inflammation found in CF. Another area of interest would be to use the serum samples collected from patients with CF and to establish whether there are any differences

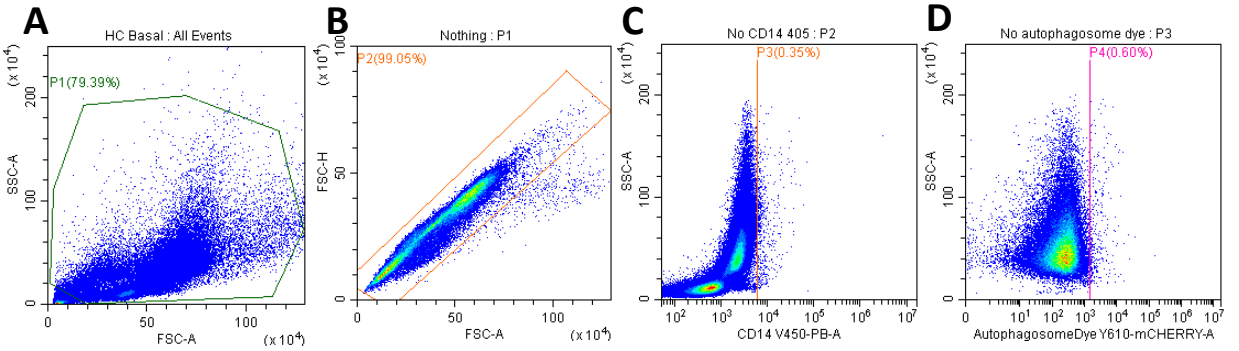
in mtDNA quantity and small extracellular vesicle with a mitochondrial signature [436, 437]. Whether CFTR modulators, over a period of 3 months, alter this would be of great interest.

Although this study failed to find any improvement of autophagy function upon the use of CFTR modulator therapies, Symdeko and ivacaftor, the development of the new triple therapies, such as Trikafta (elexacaftor, tezacaftor and ivacaftor in combination), could still be shown to improve autophagy. This is an exciting area as the new triple therapies have been reported to improve CFTR function to a greater extent than the current CFTR modulator therapies.

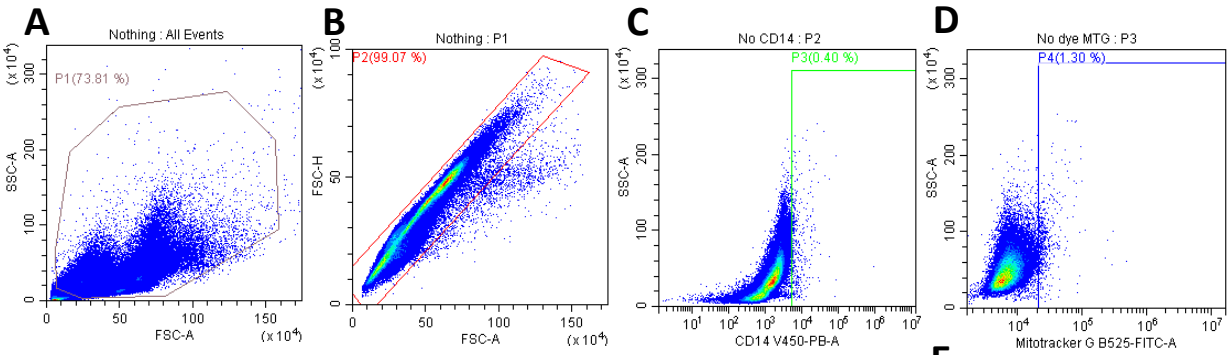
Appendix

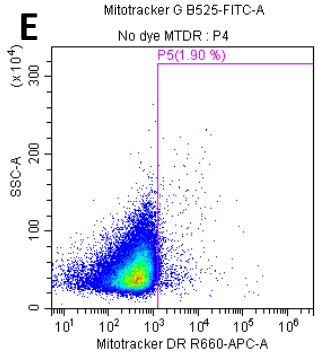


Appendix Figure 1 – Flow cytometry gating for HBECs with autophagosome dye. (A) HBECs gated based on forward and side scatter, **(B)** gated based on forward scatter area and height, **(C)** gated based on live/dead dye, and **(D)** gated based on autophagosome dye (Sigma).

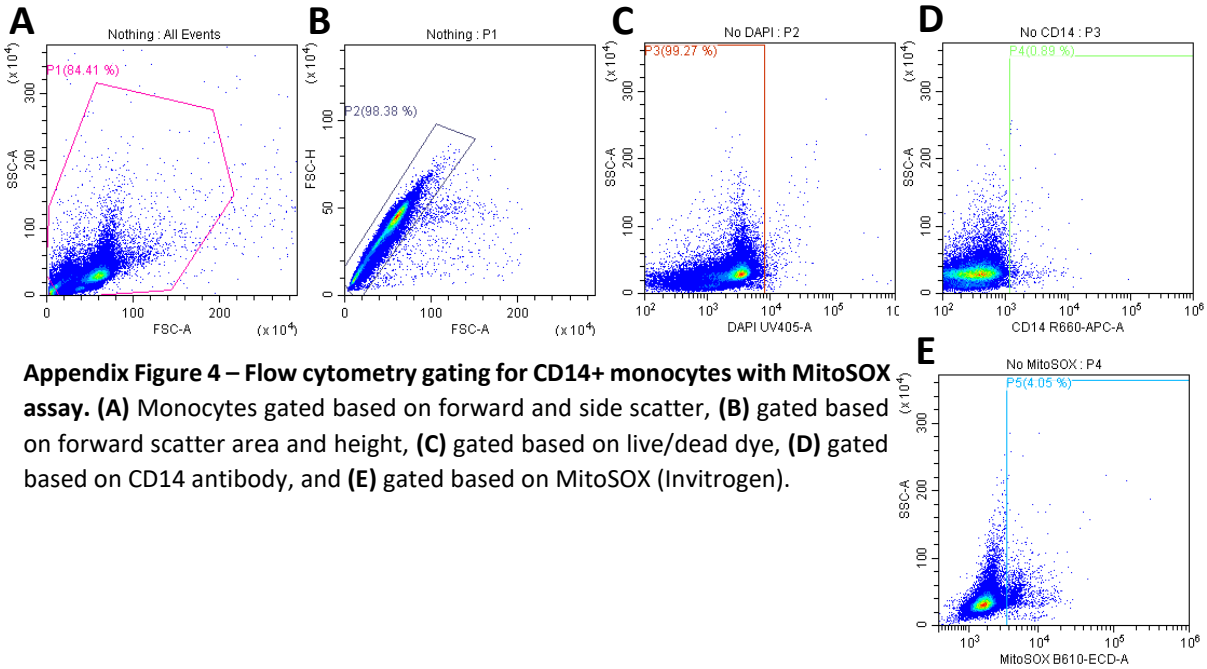


Appendix Figure 2 – Flow cytometry gating for CD14+ monocytes with autophagosome dye. (A) Monocytes gated based on forward and side scatter, **(B)** gated based on forward scatter area and height, **(C)** gated based on CD14 antibody, and **(D)** gated based on autophagosome dye (Bio-Rad).

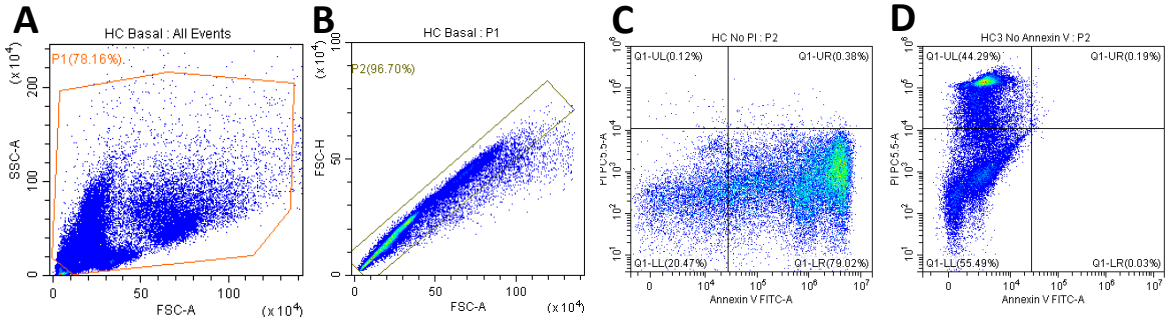




Appendix Figure 3 – Flow cytometry gating for CD14+ monocytes with MTDR and MTG. (A) Monocytes gated based on forward and side scatter, **(B)** gated based on forward scatter area and height, **(C)** gated based on CD14 antibody, **(D)** gated based on Mitotracker Green (Invitrogen), and **(E)** gated based on Mitotracker Deep Red dye (Invitrogen).



Appendix Figure 4 – Flow cytometry gating for CD14+ monocytes with MitoSOX assay. (A) Monocytes gated based on forward and side scatter, **(B)** gated based on forward scatter area and height, **(C)** gated based on live/dead dye, **(D)** gated based on CD14 antibody, and **(E)** gated based on MitoSOX (Invitrogen).



Appendix Figure 5 – Flow cytometry gating for PBMCs with annexin V live/dead assay. (A) PBMCs gated based on forward and side scatter, **(B)** gated based on forward scatter area and height, **(C)** gated based on propidium iodide, and **(D)** gated based on annexin V Alexa Fluor 488 (Thermo Fisher Scientific).

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24 May 2017

Dear Professor Peckham

Letter of HRA Approval

Study title:	**Identifying the biochemical signalling pathways involved in lung inflammation and arthropathy in patients with cystic fibrosis**
IRAS project ID:	**201570**
REC reference:	**17/YH/0084**
Sponsor	**University of Leeds**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

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Natalie Wilson
Assessor

Email: hra.approval@nhs.net

Copy to: *NHS Research Ethics Officer, University of Leeds, Sponsor contact*
Mrs Anne Gowing, Leeds Teaching Hospitals NHS Trust, Lead NHS R&D contact

References

1. Cutting GR: **Cystic fibrosis genetics: from molecular understanding to clinical application**. *Nat Rev Genet* 2015, **16**(1):45-56.
2. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL *et al*: **Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA**. *Science* 1989, **245**(4922):1066-1073.
3. Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, Rozmahel R, Cole JL, Kennedy D, Hidaka N *et al*: **Identification of the cystic fibrosis gene: chromosome walking and jumping**. *Science* 1989, **245**(4922):1059-1065.
4. Zielenski J, Rozmahel R, Bozon D, Kerem B, Grzelczak Z, Riordan JR, Rommens J, Tsui LC: **Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene**. *Genomics* 1991, **10**(1):214-228.
5. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC: **Identification of the cystic fibrosis gene: genetic analysis**. *Science* 1989, **245**(4922):1073-1080.
6. Wainwright BJ, Scambler PJ, Schmidtke J, Watson EA, Law HY, Farrall M, Cooke HJ, Eiberg H, Williamson R: **Localization of cystic fibrosis locus to human chromosome 7cen-q22**. *Nature* 1985, **318**(6044):384-385.
7. De Boeck K, Zolin A, Cuppens H, Olesen HV, Viviani L: **The relative frequency of CFTR mutation classes in European patients with cystic fibrosis**. *J Cyst Fibros* 2014, **13**(4):403-409.
8. Morral N, Bertranpetit J, Estivill X, Nunes V, Casals T, Gimenez J, Reis A, Varon-Mateeva R, Macek M, Jr., Kalaydjieva L *et al*: **The origin of the major cystic fibrosis mutation (delta F508) in European populations**. *Nat Genet* 1994, **7**(2):169-175.
9. De Boeck K, Amaral MD: **Progress in therapies for cystic fibrosis**. *Lancet Respir Med* 2016, **4**(8):662-674.
10. Kerem E, Kerem B: **The relationship between genotype and phenotype in cystic fibrosis**. *Curr Opin Pulm Med* 1995, **1**(6):450-456.
11. Lester LA, Kraut J, Lloyd-Still J, Karrison T, Mott C, Billstrand C, Lemke A, Ober C: **Delta F508 genotype does not predict disease severity in an ethnically diverse cystic fibrosis population**. *Pediatrics* 1994, **93**(1):114-118.
12. Aron Y, Polla BS, Bienvenu T, Dall'ava J, Dusser D, Hubert D: **HLA class II polymorphism in cystic fibrosis. A possible modifier of pulmonary phenotype**. *Am J Respir Crit Care Med* 1999, **159**(5 Pt 1):1464-1468.

13. Doring G, Krogh-Johansen H, Weidinger S, Hoiby N: **Allotypes of alpha 1-antitrypsin in patients with cystic fibrosis, homozygous and heterozygous for deltaF508**. *Pediatr Pulmonol* 1994, **18**(1):3-7.
14. Grasemann H, Knauer N, Buscher R, Hubner K, Drazen JM, Ratjen F: **Airway nitric oxide levels in cystic fibrosis patients are related to a polymorphism in the neuronal nitric oxide synthase gene**. *Am J Respir Crit Care Med* 2000, **162**(6):2172-2176.
15. Ratjen F, Bell SC, Rowe SM, Goss CH, Quittner AL, Bush A: **Cystic fibrosis**. *Nature Reviews Disease Primers* 2015, **1**:15010.
16. Davis PB, Drumm M, Konstan MW: **Cystic fibrosis**. *Am J Respir Crit Care Med* 1996, **154**(5):1229-1256.
17. Chmiel JF, Berger M, Konstan MW: **The role of inflammation in the pathophysiology of CF lung disease**. *Clin Rev Allergy Immunol* 2002, **23**(1):5-27.
18. Dong YJ, Chao AC, Kouyama K, Hsu YP, Bocian RC, Moss RB, Gardner P: **Activation of CFTR chloride current by nitric oxide in human T lymphocytes**. *Embo j* 1995, **14**(12):2700-2707.
19. Weyler RT, Yurko-Mauro KA, Rubenstein R, Kollen WJ, Reenstra W, Altschuler SM, Egan M, Mulberg AE: **CFTR is functionally active in GnRH-expressing GT1-7 hypothalamic neurons**. *Am J Physiol* 1999, **277**(3 Pt 1):C563-571.
20. Cant N, Pollock N, Ford RC: **CFTR structure and cystic fibrosis**. *Int J Biochem Cell Biol* 2014, **52**:15-25.
21. Serohijos AW, Hegedus T, Aleksandrov AA, He L, Cui L, Dokholyan NV, Riordan JR: **Phenylalanine-508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D structure crucial to assembly and channel function**. *Proc Natl Acad Sci U S A* 2008, **105**(9):3256-3261.
22. Valdivieso AG, Santa-Coloma TA: **CFTR activity and mitochondrial function**. *Redox Biol* 2013, **1**:190-202.
23. Broadbent SD, Ramjeesingh M, Bear CE, Argent BE, Linsdell P, Gray MA: **The cystic fibrosis transmembrane conductance regulator is an extracellular chloride sensor**. *Pflugers Arch* 2015, **467**(8):1783-1794.
24. Mendoza JL, Schmidt A, Li Q, Nuvaga E, Barrett T, Bridges RJ, Feranchak AP, Brautigam CA, Thomas PJ: **Requirements for efficient correction of DeltaF508 CFTR revealed by analyses of evolved sequences**. *Cell* 2012, **148**(1-2):164-174.
25. Rabeh WM, Bossard F, Xu H, Okiyoneda T, Bagdany M, Mulvihill CM, Du K, di Bernardo S, Liu Y, Konermann L *et al*: **Correction of both NBD1 energetics and domain interface is required to restore DeltaF508 CFTR folding and function**. *Cell* 2012, **148**(1-2):150-163.

26. Okiyoneda T, Barriere H, Bagdany M, Rabeh WM, Du K, Hohfeld J, Young JC, Lukacs GL: Peripheral protein quality control removes unfolded CFTR from the plasma membrane. *Science* 2010, 329(5993):805-810.
27. Hull J: Cystic fibrosis transmembrane conductance regulator dysfunction and its treatment. In: *J R Soc Med.* vol. 105; 2012: S2-8.
28. Scambler T, Jarosz-Griffiths HH, Lara-Reyna S, Pathak S, Wong C, Holbrook J, Martinon F, Savic S, Peckham D, McDermott MF: ENaC-mediated sodium influx exacerbates NLRP3-dependent inflammation in cystic fibrosis. *Elife* 2019, 8.
29. Riordan JR: CFTR function and prospects for therapy. *Annu Rev Biochem* 2008, 77:701-726.
30. Zhou Z, Duerr J, Johannesson B, Schubert SC, Treis D, Harm M, Graeber SY, Dalpke A, Schultz C, Mall MA: The ENaC-overexpressing mouse as a model of cystic fibrosis lung disease. *J Cyst Fibros* 2011, 10 Suppl 2:S172-182.
31. Clary-Meinesz C, Mouroux J, Cosson J, Huitorel P, Blaive B: Influence of external pH on ciliary beat frequency in human bronchi and bronchioles. *Eur Respir J* 1998, 11(2):330-333.
32. Allen DB, Maguire JJ, Mahdavian M, Wicke C, Marcocci L, Scheuenstuhl H, Chang M, Le AX, Hopf HW, Hunt TK: Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms. *Arch Surg* 1997, 132(9):991-996.
33. Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, Karp PH, Wohlford-Lenane CL, Haagsman HP, van Eijk M *et al*: Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature* 2012, 487(7405):109-113.
34. Foundation CF: Patient Registry Annual Data Report. 2016.
35. Amin R, Dupuis A, Aaron SD, Ratjen F: The effect of chronic infection with Aspergillus fumigatus on lung function and hospitalization in patients with cystic fibrosis. *Chest* 2010, 137(1):171-176.
36. Wark PA, Tooze M, Cheese L, Whitehead B, Gibson PG, Wark KF, McDonald VM: Viral infections trigger exacerbations of cystic fibrosis in adults and children. *Eur Respir J* 2012, 40(2):510-512.
37. Kunzelmann K, Mehta A: CFTR: a hub for kinases and crosstalk of cAMP and Ca2+. *Febs j* 2013, 280(18):4417-4429.
38. Patel W, Moore PJ, Sassano MF, Lopes-Pacheco M, Aleksandrov AA, Amaral MD, Tarran R, Gray MA: Increases in cytosolic Ca2+ induce dynamin- and calcineurin-dependent internalisation of CFTR. *Cell Mol Life Sci* 2019, 76(5):977-994.
39. Bonfield TL, Hodges CA, Cotton CU, Drumm ML: Absence of the cystic fibrosis transmembrane regulator (Cftr) from myeloid-derived cells slows

resolution of inflammation and infection. *J Leukoc Biol* 2012, **92**(5):1111-1122.
40. Robledo-Avila FH, Ruiz-Rosado JD, Brockman KL, Kopp BT, Amer AO, McCoy K, Bakaletz LO, Partida-Sanchez S: **Dysregulated Calcium Homeostasis in Cystic Fibrosis Neutrophils Leads to Deficient Antimicrobial Responses**. *J Immunol* 2018, **201**(7):2016-2027.
41. Pier GB, Grout M, Zaidi T, Meluleni G, Mueschenborn SS, Banting G, Ratcliff R, Evans MJ, Colledge WH: **Salmonella typhi uses CFTR to enter intestinal epithelial cells**. *Nature* 1998, **393**(6680):79-82.
42. Ajonuma LC, Fok KL, Ho LS, Chan PK, Chow PH, Tsang LL, Wong CH, Chen J, Li S, Rowlands DK *et al*: **CFTR is required for cellular entry and internalization of Chlamydia trachomatis**. *Cell Biol Int* 2010, **34**(6):593-600.
43. Pier GB: **Role of the cystic fibrosis transmembrane conductance regulator in innate immunity to Pseudomonas aeruginosa infections**. *Proc Natl Acad Sci U S A* 2000, **97**(16):8822-8828.
44. Farinha CM, Matos P, Amaral MD: **Control of cystic fibrosis transmembrane conductance regulator membrane trafficking: not just from the endoplasmic reticulum to the Golgi**. *Febs j* 2013, **280**(18):4396-4406.
45. Skach WR: **Defects in processing and trafficking of the cystic fibrosis transmembrane conductance regulator**. *Kidney Int* 2000, **57**(3):825-831.
46. Amaral MD: **CFTR and chaperones: processing and degradation**. *J Mol Neurosci* 2004, **23**(1-2):41-48.
47. Coppinger JA, Hutt DM, Razvi A, Koulov AV, Pankow S, Yates JR, 3rd, Balch WE: **A chaperone trap contributes to the onset of cystic fibrosis**. *PLoS One* 2012, **7**(5):e37682.
48. Scott-Ward TS, Amaral MD: **Deletion of Phe508 in the first nucleotide-binding domain of the cystic fibrosis transmembrane conductance regulator increases its affinity for the heat shock cognate 70 chaperone**. *Febs j* 2009, **276**(23):7097-7109.
49. Nishimura N, Balch WE: **A di-acidic signal required for selective export from the endoplasmic reticulum**. *Science* 1997, **277**(5325):556-558.
50. Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE: **Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis**. *Cell* 1990, **63**(4):827-834.
51. Rojas AM, Fuentes G, Rausell A, Valencia A: **The Ras protein superfamily: evolutionary tree and role of conserved amino acids**. *J Cell Biol* 2012, **196**(2):189-201.

52. Swiatecka-Urban A, Talebian L, Kanno E, Moreau-Marquis S, Coutermarsh B, Hansen K, Karlson KH, Barnaby R, Cheney RE, Langford GM *et al*: Myosin Vb is required for trafficking of the cystic fibrosis transmembrane conductance regulator in Rab11a-specific apical recycling endosomes in polarized human airway epithelial cells. *J Biol Chem* 2007, **282**(32):23725-23736.
53. Venerando A, Franchin C, Cant N, Cozza G, Pagano MA, Tosoni K, Al-Zahrani A, Arrigoni G, Ford RC, Mehta A *et al*: Detection of phospho-sites generated by protein kinase CK2 in CFTR: mechanistic aspects of Thr1471 phosphorylation. *PLoS One* 2013, **8**(9):e74232.
54. Yu H, Burton B, Huang CJ, Worley J, Cao D, Johnson JP, Jr., Urrutia A, Joubran J, Seepersaud S, Sussky K *et al*: Ivacaftor potentiation of multiple CFTR channels with gating mutations. *J Cyst Fibros* 2012, **11**(3):237-245.
55. De Boeck K, Munck A, Walker S, Faro A, Hiatt P, Gilmartin G, Higgins M: Efficacy and safety of ivacaftor in patients with cystic fibrosis and a non-G551D gating mutation. *J Cyst Fibros* 2014, **13**(6):674-680.
56. Moss RB, Flume PA, Elborn JS, Cooke J, Rowe SM, McColley SA, Rubenstein RC, Higgins M: Efficacy and safety of ivacaftor in patients with cystic fibrosis who have an Arg117His-CFTR mutation: a double-blind, randomised controlled trial. *Lancet Respir Med* 2015, **3**(7):524-533.
57. Wainwright CE, Elborn JS, Ramsey BW: Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N Engl J Med* 2015, **373**(18):1783-1784.
58. Connett G: Lumacaftor-ivacaftor in the treatment of cystic fibrosis: design, development and place in therapy. *Drug Des Devel Ther* 2019, **13**:2405-2412.
59. Jennings MT, Dezube R, Paranjape S, West NE, Hong G, Braun A, Grant J, Merlo CA, Lechtzin N: An Observational Study of Outcomes and Tolerances in Patients with Cystic Fibrosis Initiated on Lumacaftor/Ivacaftor. *Ann Am Thorac Soc* 2017, **14**(11):1662-1666.
60. Hubert D, Chiron R, Camara B, Grenet D, Prevotat A, Bassinet L, Dominique S, Rault G, Macey J, Honore I *et al*: Real-life initiation of lumacaftor/ivacaftor combination in adults with cystic fibrosis homozygous for the Phe508del CFTR mutation and severe lung disease. *J Cyst Fibros* 2017, **16**(3):388-391.
61. Taylor-Cousar JL, Munck A, McKone EF, van der Ent CK, Moeller A, Simard C, Wang LT, Ingenito EP, McKee C, Lu Y *et al*: Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del. *N Engl J Med* 2017, **377**(21):2013-2023.

62. Lommatzsch ST, Taylor-Cousar JL: The combination of tezacaftor and ivacaftor in the treatment of patients with cystic fibrosis: clinical evidence and future prospects in cystic fibrosis therapy. *Ther Adv Respir Dis* 2019, 13.
63. Jarosz-Griffiths HH, Scambler T, Wong CH, Lara-Reyna S, Holbrook J, Martinon F, Savic S, Whitaker P, Etherington C, Spoletini G *et al*: Different CFTR modulator combinations downregulate inflammation differently in cystic fibrosis. *Elife* 2020, 9.
64. Keating D, Marigowda G, Burr L, Daines C, Mall MA, McKone EF, Ramsey BW, Rowe SM, Sass LA, Tullis E *et al*: VX-445-Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. *N Engl J Med* 2018, 379(17):1612-1620.
65. Davies JC, Moskowitz SM, Brown C, Horsley A, Mall MA, McKone EF, Plant BJ, Prais D, Ramsey BW, Taylor-Cousar JL *et al*: VX-659-Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. *N Engl J Med* 2018, 379(17):1599-1611.
66. Awatade NT, Ramalho S, Silva IAL, Felicio V, Botelho HM, de Poel E, Vonk A, Beekman JM, Farinha CM, Amaral MD: R560S: A class II CFTR mutation that is not rescued by current modulators. *J Cyst Fibros* 2019, 18(2):182-189.
67. Carlile GW, Yang Q, Matthes E, Liao J, Radinovic S, Miyamoto C, Robert R, Hanrahan JW, Thomas DY: A novel triple combination of pharmacological chaperones improves F508del-CFTR correction. *Sci Rep* 2018, 8(1):11404.
68. Sorio C, Buffelli M, Angiari C, Ettorre M, Johansson J, Vezzalini M, Viviani L, Ricciardi M, Verzè G, Assael BM *et al*: Defective CFTR Expression and Function Are Detectable in Blood Monocytes: Development of a New Blood Test for Cystic Fibrosis. *PLoS One* 2011, 6(7).
69. Jundi B, Pohl K, McElvaney, Reeves E: The importance of CFTR expression for neutrophil function in patients with Cystic Fibrosis. *BMC Proc* 2015, 9(Suppl 1):A36.
70. Painter RG, Valentine VG, Lanson NA, Jr., Leidal K, Zhang Q, Lombard G, Thompson C, Viswanathan A, Nauseef WM, Wang G: CFTR Expression in human neutrophils and the phagolysosomal chlorination defect in cystic fibrosis. *Biochemistry* 2006, 45(34):10260-10269.
71. McDonald TV, Nghiem PT, Gardner P, Martens CL: Human lymphocytes transcribe the cystic fibrosis transmembrane conductance regulator gene and exhibit CF-defective cAMP-regulated chloride current. *J Biol Chem* 1992, 267(5):3242-3248.
72. Gao Z, Su X: CFTR regulates acute inflammatory responses in macrophages. *Qjm* 2015, 108(12):951-958.

73. Porto PD, Cifani N, Guarnieri S, Di Domenico EG, Mariggiò MA, Spadaro F, Guglietta S, Anile M, Venuta F, Quattrucci S *et al*: Dysfunctional CFTR Alters the Bactericidal Activity of Human Macrophages against Pseudomonas aeruginosa. *PLoS One* 2011, 6(5).
74. Painter RG, Bonvillain RW, Valentine VG, Lombard GA, LaPlace SG, Nauseef WM, Wang G: The role of chloride anion and CFTR in killing of Pseudomonas aeruginosa by normal and CF neutrophils. *J Leukoc Biol* 2008, 83(6):1345-1353.
75. Xu Y, Krause A, Hamai H, Harvey BG, Worgall TS, Worgall S: Proinflammatory phenotype and increased caveolin-1 in alveolar macrophages with silenced CFTR mRNA. *PLoS One* 2010, 5(6):e11004.
76. Xu Y, Tertilt C, Krause A, Quadri LE, Crystal RG, Worgall S: Influence of the cystic fibrosis transmembrane conductance regulator on expression of lipid metabolism-related genes in dendritic cells. *Respir Res* 2009, 10:26.
77. Hampton TH, Stanton BA: A novel approach to analyze gene expression data demonstrates that the DeltaF508 mutation in CFTR downregulates the antigen presentation pathway. *Am J Physiol Lung Cell Mol Physiol* 2010, 298(4):L473-482.
78. Lara-Reyna S, Holbrook J, Jarosz-Griffiths H, Peckham D, McDermott MF,: Dysregulated Signalling Pathways in Innate Immune Cells With Cystic Fibrosis Mutations. *Cellular and molecular life sciences : CMLS* 2020.
79. Luciani A, Villella VR, Esposito S, Brunetti-Pierri N, Medina D, Settembre C, Gavina M, Pulze L, Giardino I, Pettoello-Mantovani M *et al*: Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. *Nat Cell Biol* 2010, 12(9):863-875.
80. Ribeiro CM, Boucher RC: Role of endoplasmic reticulum stress in cystic fibrosis-related airway inflammatory responses. *Proc Am Thorac Soc* 2010, 7(6):387-394.
81. Mott LS, Park J, Murray CP, Gangell CL, de Klerk NH, Robinson PJ, Robertson CF, Ranganathan SC, Sly PD, Stick SM: Progression of early structural lung disease in young children with cystic fibrosis assessed using CT. *Thorax* 2012, 67(6):509-516.
82. Livraghi-Butrico A, Kelly EJ, Klem ER, Dang H, Wolfgang MC, Boucher RC, Randell SH, O'Neal WK: Mucus clearance, MyD88-dependent and MyD88-independent immunity modulate lung susceptibility to spontaneous bacterial infection and inflammation. *Mucosal Immunol* 2012, 5(4):397-408.

83. Bonfield TL, Panuska JR, Konstan MW, Hilliard KA, Hilliard JB, Ghnaim H, Berger M: Inflammatory cytokines in cystic fibrosis lungs. *Am J Respir Crit Care Med* 1995, 152(6 Pt 1):2111-2118.
84. Koller DY, Nething I, Otto J, Urbanek R, Eichler I: Cytokine concentrations in sputum from patients with cystic fibrosis and their relation to eosinophil activity. *Am J Respir Crit Care Med* 1997, 155(3):1050-1054.
85. Moser C, Jensen PO, Pressler T, Frederiksen B, Lanng S, Kharazmi A, Koch C, Hoiby N: Serum concentrations of GM-CSF and G-CSF correlate with the Th1/Th2 cytokine response in cystic fibrosis patients with chronic Pseudomonas aeruginosa lung infection. *Apmis* 2005, 113(6):400-409.
86. Bouchon, Dietrich J, Colona M: Cutting Edge: Inflammatory Responses Can Be Triggered by TREM-1, a Novel Receptor Expressed on Neutrophils and Monocytes. *Journal of immunology (Baltimore, Md : 1950)* 2000, 164(10).
87. Forrester DL, Barr HL, Fogarty A, Knox A: sTREM-1 Is Elevated in Cystic Fibrosis and Correlates With Proteases. *Pediatric pulmonology* 2017, 52(4).
88. Masekela R, Anderson R, de Boeck K, Vreys M, Steel HC, Olurunju S, Grenn RJ: Expression of Soluble Triggering Receptor Expressed on Myeloid cells-1 in Childhood CF and non-CF Bronchiectasis. *Pediatric pulmonology* 2015, 50(4).
89. Karp CL, Flick LM, Park KW, Softic S, Greer TM, Keledjian R, Yang R, Uddin J, Guggino WB, Atabani SF *et al*: Defective lipoxin-mediated anti-inflammatory activity in the cystic fibrosis airway. *Nat Immunol* 2004, 5(4):388-392.
90. Grasemann H, Michler E, Wallot M, Ratjen F: Decreased concentration of exhaled nitric oxide (NO) in patients with cystic fibrosis. *Pediatr Pulmonol* 1997, 24(3):173-177.
91. Saba S, Soong G, Greenberg S, Prince A: Bacterial stimulation of epithelial G-CSF and GM-CSF expression promotes PMN survival in CF airways. *Am J Respir Cell Mol Biol* 2002, 27(5):561-567.
92. McElvaney OJ, Gunaratnam C, O'Neill L, Reeves EP, McElvaney G: Metabolic Reprogramming of the Cystic Fibrosis Neutrophil Drives Interleukin-1β via the NLRP3 Inflammasome. *Pediatric Pulmonology* 2018, 53:S176.
93. Downey DG, Martin SL, Dempster M, Moore JE, Keogan MT, Starcher B, Edgar J, Bilton D, Elborn JS: The relationship of clinical and inflammatory markers to outcome in stable patients with cystic fibrosis. *Pediatr Pulmonol* 2007, 42(3):216-220.

94. Marcos V, Zhou-Suckow Z, Onder Yildirim A, Bohla A, Hector A, Vitkov L, Krautgartner WD, Stoiber W, Griese M, Eickelberg O *et al*: Free DNA in cystic fibrosis airway fluids correlates with airflow obstruction. *Mediators Inflamm* 2015, 2015:408935.
95. Gehrig S, Duerr J, Weitnauer M, Wagner CJ, Graeber SY, Schatterny J, Hirtz S, Belaaouaj A, Dalpke AH, Schultz C *et al*: Lack of neutrophil elastase reduces inflammation, mucus hypersecretion, and emphysema, but not mucus obstruction, in mice with cystic fibrosis-like lung disease. *Am J Respir Crit Care Med* 2014, 189(9):1082-1092.
96. Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, Brain JD, Accurso FJ, Henson PM: Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *J Clin Invest* 2002, 109(5):661-670.
97. Ng HP, Zhou Y, Song K, Hodges CA, Drumm ML, Wang G: Neutrophil-mediated phagocytic host defense defect in myeloid Cftr-inactivated mice. *PLoS One* 2014, 9(9):e106813.
98. Berger M, Sorensen RU, Tosi MF, Dearborn DG, Doring G: Complement receptor expression on neutrophils at an inflammatory site, the Pseudomonas-infected lung in cystic fibrosis. *J Clin Invest* 1989, 84(4):1302-1313.
99. Tabary O, Corvol H, Boncoeur E, Chadelat K, Fitting C, Cavaillon JM, Clement A, Jacquot J: Adherence of airway neutrophils and inflammatory response are increased in CF airway epithelial cell-neutrophil interactions. *Am J Physiol Lung Cell Mol Physiol* 2006, 290(3):L588-596.
100. Gray RD, Hardisty G, Regan KH, Smith M, Robb CT, Duffin R, Mackellar A, Felton JM, Paemka L, McCullagh BN *et al*: Delayed neutrophil apoptosis enhances NET formation in cystic fibrosis. *Thorax* 2018, 73(2):134-144.
101. Tarique AA, Sly PD, Cardenas DG, Luo L, Stow JL, Bell SC, Wainwright CE, Fantino E: Differential expression of genes and receptors in monocytes from patients with cystic fibrosis. *J Cyst Fibros* 2019, 18(3):342-348.
102. Sturges NC, Wikstrom ME, Winfield KR, Gard SE, Brennan S, Sly PD, Upham JW: Monocytes from children with clinically stable cystic fibrosis show enhanced expression of Toll-like receptor 4. *Pediatr Pulmonol* 2010, 45(9):883-889.
103. Lara-Reyna S, Scambler T, Holbrook J, Wong C, Jarosz-Griffiths HH, Martinon F, Savic S, Peckham D, McDermott MF: Metabolic Reprograming of Cystic Fibrosis Macrophages via the IRE1alpha Arm of the Unfolded Protein Response Results in Exacerbated Inflammation. *Front Immunol* 2019, 10:1789.

104. Zaman MM, Gelrud A, Junaidi O, Regan MM, Warny M, Shea JC, Kelly C, O'Sullivan BP, Freedman SD: Interleukin 8 secretion from monocytes of subjects heterozygous for the deltaF508 cystic fibrosis transmembrane conductance regulator gene mutation is altered. *Clin Diagn Lab Immunol* 2004, 11(5):819-824.
105. Alexis NE, Muhlebach MS, Peden DB, Noah TL: Attenuation of host defense function of lung phagocytes in young cystic fibrosis patients. *J Cyst Fibros* 2006, 5(1):17-25.
106. Rao S, Wright AK, Montiero W, Ziegler-Heitbrock L, Grigg J: Monocyte chemoattractant chemokines in cystic fibrosis. *J Cyst Fibros* 2009, 8(2):97-103.
107. McKiernan PJ, Molloy KP, Cryan SA, McElvaney NG, Greene CM: X Chromosome-encoded MicroRNAs Are Functionally Increased in Cystic Fibrosis Monocytes. *Am J Respir Crit Care Med* 2018, 197(5):668-670.
108. Avendano-Ortiz J, Llanos-Gonzalez E, Toledano V, Del Campo R, Cubillos-Zapata C, Lozano-Rodriguez R, Ismail A, Prados C, Gomez-Campelo P, Aguirre LA *et al*: Pseudomonas aeruginosa colonization causes PD-L1 overexpression on monocytes, impairing the adaptive immune response in patients with cystic fibrosis. *J Cyst Fibros* 2019, 18(5):630-635.
109. Velard F, Jourdain ML, Abdallah D, Jacquot J, Ronan N, McCarthy Y, Flanagan E, Plant B: Overexpression of RANK and M-CSFR in Monocytes of G551D-Bearing Patients with Cystic Fibrosis. *Am J Respir Crit Care Med* 2018, 198(7):968-970.
110. Hisert KB, Schoenfelt KQ, Cooke G, Grogan B, Launspach JL, Gallagher CG, Donnelly SC, Welsh MJ, Singh PK, McKone EF *et al*: Ivacaftor-Induced Proteomic Changes Suggest Monocyte Defects May Contribute to the Pathogenesis of Cystic Fibrosis. *Am J Respir Cell Mol Biol* 2016, 54(4):594-597.
111. Lamothe J, Valvano MA: Burkholderia cenocepacia-induced delay of acidification and phagolysosomal fusion in cystic fibrosis transmembrane conductance regulator (CFTR)-defective macrophages. *Microbiology* 2008, 154(Pt 12):3825-3834.
112. Di A, Brown ME, Deriy LV, Li C, Szeto FL, Chen Y, Huang P, Tong J, Naren AP, Bindokas V *et al*: CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. *Nat Cell Biol* 2006, 8(9):933-944.
113. Barriere H, Bagdany M, Bossard F, Okiyoneda T, Wojewodka G, Gruenert D, Radzioch D, Lukacs GL: Revisiting the role of cystic fibrosis transmembrane conductance regulator and counterion permeability in

the pH regulation of endocytic organelles. *Mol Biol Cell* 2009, 20(13):3125-3141.
114. Haggie PM, Verkman AS: Cystic fibrosis transmembrane conductance regulator-independent phagosomal acidification in macrophages. *J Biol Chem* 2007, 282(43):31422-31428.
115. Bruscia EM, Zhang PX, Ferreira E, Caputo C, Emerson JW, Tuck D, Krause DS, Egan ME: Macrophages directly contribute to the exaggerated inflammatory response in cystic fibrosis transmembrane conductance regulator-/- mice. *Am J Respir Cell Mol Biol* 2009, 40(3):295-304.
116. Tarique AA, Sly PD, Holt PG, Bosco A, Ware RS, Logan J, Bell SC, Wainwright CE, Fantino E: CFTR-dependent defect in alternatively-activated macrophages in cystic fibrosis. *J Cyst Fibros* 2017, 16(4):475-482.
117. Lubamba BA, Jones LC, O'Neal WK, Boucher RC, Ribeiro CM: X-Box-Binding Protein 1 and Innate Immune Responses of Human Cystic Fibrosis Alveolar Macrophages. *Am J Respir Crit Care Med* 2015, 192(12):1449-1461.
118. Zhang S, Shrestha CL, Kopp BT: Cystic fibrosis transmembrane conductance regulator (CFTR) modulators have differential effects on cystic fibrosis macrophage function. *Sci Rep* 2018, 8(1):17066.
119. Venkatakrishnan A, Stecenko AA, King G, Blackwell TR, Brigham KL, Christman JW, Blackwell TS: Exaggerated activation of nuclear factor-kappaB and altered IkappaB-beta processing in cystic fibrosis bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2000, 23(3):396-403.
120. Weber AJ, Soong G, Bryan R, Saba S, Prince A: Activation of NF-kappaB in airway epithelial cells is dependent on CFTR trafficking and Cl- channel function. *Am J Physiol Lung Cell Mol Physiol* 2001, 281(1):L71-78.
121. Tabary O, Boncoeur E, de Martin R, Pepperkok R, Clement A, Schultz C, Jacquot J: Calcium-dependent regulation of NF-(kappa)B activation in cystic fibrosis airway epithelial cells. *Cell Signal* 2006, 18(5):652-660.
122. Ribeiro CM, Paradiso AM, Carew MA, Shears SB, Boucher RC: Cystic fibrosis airway epithelial Ca2+ i signaling: the mechanism for the larger agonist-mediated Ca2+ i signals in human cystic fibrosis airway epithelia. *J Biol Chem* 2005, 280(11):10202-10209.
123. Ribeiro CM, Paradiso AM, Schwab U, Perez-Vilar J, Jones L, O'Neal W, Boucher RC: Chronic airway infection/inflammation induces a Ca2+i-dependent hyperinflammatory response in human cystic fibrosis airway epithelia. *J Biol Chem* 2005, 280(18):17798-17806.
124. Aldallal N, McNaughton EE, Manzel LJ, Richards AM, Zabner J, Ferkol TW, Look DC: Inflammatory response in airway epithelial cells isolated from

patients with cystic fibrosis. *Am J Respir Crit Care Med* 2002, **166**(9):1248-1256.
125. Scambler T. Holbrook J, Savic S., Peckham D., McDermott M.: Autoinflammatory diseases of the lung. *Journal of Immunology* 2018 (Under review).
126. Pongnimitprasert N, Hurtado M, Lamari F, El Benna J, Dupuy C, Fay M, Foglietti MJ, Bernard M, Gougerot-Pocidalo MA, Braut-Boucher F: Implication of NADPH Oxidases in the Early Inflammation Process Generated by Cystic Fibrosis Cells. *ISRN Inflamm* 2012, **2012**.
127. Tang A, Sharma A, Jen R, Hirschfeld AF, Chilvers MA, Lavoie PM, Turvey SE: Inflammasome-mediated IL-1beta production in humans with cystic fibrosis. *PLoS One* 2012, **7**(5):e37689.
128. Deshayes S, Georgin-Lavialle S, Hot A, Durel CA, Hachulla E, Rouanes N, Audia S, Le Gallou T, Quartier P, Urbanski G *et al*: Efficacy of Continuous Interleukin 1 Blockade in Mevalonate Kinase Deficiency: A Multicenter Retrospective Study in 13 Adult Patients and Literature Review. *J Rheumatol* 2018, **45**(3):425-429.
129. van der Hilst J, Moutschen M, Messiaen PE, Lauwerys BR, Vanderschueren S: Efficacy of anti-IL-1 treatment in familial Mediterranean fever: a systematic review of the literature. *Biologics* 2016, **10**:75-80.
130. La Torre F, Muratore M, Vitale A, Moramarco F, Quarta L, Cantarini L: Canakinumab efficacy and long-term tocilizumab administration in tumor necrosis factor receptor-associated periodic syndrome (TRAPS). *Rheumatol Int* 2015, **35**(11):1943-1947.
131. Goldbach-Mansky R, Dailey NJ, Canna SW, Gelabert A, Jones J, Rubin BI, Kim HJ, Brewer C, Zalewski C, Wiggs E *et al*: Neonatal-onset multisystem inflammatory disease responsive to interleukin-1beta inhibition. *N Engl J Med* 2006, **355**(6):581-592.
132. Lovell DJ, Bowyer SL, Solinger AM: Interleukin-1 blockade by anakinra improves clinical symptoms in patients with neonatal-onset multisystem inflammatory disease. *Arthritis Rheum* 2005, **52**(4):1283-1286.
133. Latz E, Xiao TS, Stutz A: Activation and regulation of the inflammasomes. *Nat Rev Immunol* 2013, **13**(6):397-411.
134. Martinon F, Burns K, Tschopp J: The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002, **10**(2):417-426.
135. Iannitti RG, Napolioni V, Oikonomou V, De Luca A, Galosi C, Pariano M, Massi-Benedetti C, Borghi M, Puccetti M, Lucidi V *et al*: IL-1 receptor

antagonist ameliorates inflammasome-dependent inflammation in murine and human cystic fibrosis. *Nat Commun* 2016, **7**:10791.
136. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B: TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006, **24**(2):179-189.
137. Harrison OJ, Srinivasan N, Pott J, Schiering C, Krausgruber T, Ilott NE, Maloy KJ: Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3(+) Treg cell function in the intestine. *Mucosal Immunol* 2015, **8**(6):1226-1236.
138. Rolfes V, Ribeiro LS, Hawwari I, Bottcher L, Rosero N, Maaswerd S, Santos MLS, Prochnicki T, Silva CMdS, Wanderley CWdS *et al*: Platelets Fuel the Inflammasome Activation of Innate Immune Cells. *Cell reports* 2020, **31**(6).
139. Rimessi A, Bezzerri V, Patergnani S, Marchi S, Cabrini G, Pinton P: Mitochondrial Ca2+-dependent NLRP3 activation exacerbates the Pseudomonas aeruginosa-driven inflammatory response in cystic fibrosis. *Nat Commun* 2015, **6**:6201.
140. Moore PJ, Tarran R: The epithelial sodium channel (ENaC) as a therapeutic target for cystic fibrosis lung disease. *Expert Opin Ther Targets* 2018, **22**(8):687-701.
141. Kim CS, Ahmad S, Wu T, Walton WG, Redinbo MR, Tarran R: SPLUNC1 is an allosteric modulator of the epithelial sodium channel. *Faseb j* 2018, **32**(5):2478-2491.
142. Peckham D, Scambler T, Savic S, McDermott MF: The burgeoning field of innate immune-mediated disease and autoinflammation. *J Pathol* 2017, **241**(2):123-139.
143. Knutsen AP, Mueller KR, Hutcheson PS, Slavin RG: T- and B-cell dysregulation of IgE synthesis in cystic fibrosis patients with allergic bronchopulmonary aspergillosis. *Clin Immunol Immunopathol* 1990, **55**(1):129-138.
144. Dubin PJ, McAllister F, Kolls JK: Is cystic fibrosis a TH17 disease? *Inflamm Res* 2007, **56**(6):221-227.
145. Tiringer K, Treis A, Fucik P, Gona M, Gruber S, Renner S, Dehlink E, Nachbaur E, Horak F, Jaksch P *et al*: A Th17- and Th2-skewed cytokine profile in cystic fibrosis lungs represents a potential risk factor for Pseudomonas aeruginosa infection. *Am J Respir Crit Care Med* 2013, **187**(6):621-629.
146. Moss RB, Bocian RC, Hsu YP, Dong YJ, Kemna M, Wei T, Gardner P: Reduced IL-10 secretion by CD4+ T lymphocytes expressing mutant cystic

fibrosis transmembrane conductance regulator (CFTR). *Clin Exp Immunol* 1996, 106(2):374-388.
147. Hoffman HM, Rosengren S, Boyle DL, Cho JY, Nayar J, Mueller JL, Anderson JP, Wanderer AA, Firestein GS: Prevention of cold-associated acute inflammation in familial cold autoinflammatory syndrome by interleukin-1 receptor antagonist. *Lancet* 2004, 364(9447):1779-1785.
148. Hawkins PN, Lachmann HJ, McDermott MF: Interleukin-1-receptor antagonist in the Muckle-Wells syndrome. *N Engl J Med* 2003, 348(25):2583-2584.
149. Frenkel J, Rijkers GT, Mandey SH, Buurman SW, Houten SM, Wanders RJ, Waterham HR, Kuis W: Lack of isoprenoid products raises ex vivo interleukin-1beta secretion in hyperimmunoglobulinemia D and periodic fever syndrome. *Arthritis Rheum* 2002, 46(10):2794-2803.
150. de Koning HD, Bodar EJ, Simon A, van der Hilst JC, Netea MG, van der Meer JW: Beneficial response to anakinra and thalidomide in Schnitzler's syndrome. *Ann Rheum Dis* 2006, 65(4):542-544.
151. Gattorno M, Obici L, Cattalini M, Tormey V, Abrams K, Davis N, Speziale A, Bhansali SG, Martini A, Lachmann HJ: Canakinumab treatment for patients with active recurrent or chronic TNF receptor-associated periodic syndrome (TRAPS): an open-label, phase II study. *Ann Rheum Dis* 2017, 76(1):173-178.
152. McGonagle D, McDermott MF: A Proposed Classification of the Immunological Diseases. *PLoS Med* 2006, 3(8).
153. Bernot A, da Silva C, Petit JL, Cruaud C, Caloustian C, Castet V, Ahmed-Arab M, Dross C, Dupont M, Cattan D *et al*: Non-founder mutations in the MEFV gene establish this gene as the cause of familial Mediterranean fever (FMF). *Hum Mol Genet* 1998, 7(8):1317-1325.
154. Bernot A, Clepet C, Dasilva C, Devaud C, *et al*: A candidate gene for familial Mediterranean fever. *Nat Genet* 1997, 17(1):25-31.
155. Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. The International FMF Consortium. *Cell* 1997, 90(4):797-807.
156. Gao W, Yang J, Liu W, Wang Y, Shao F: Site-specific phosphorylation and microtubule dynamics control Pyrin inflammasome activation. *Proc Natl Acad Sci U S A* 2016, 113(33):E4857-4866.
157. Xu H, Yang J, Gao W, Li L, Li P, Zhang L, Gong YN, Peng X, Xi JJ, Chen S *et al*: Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. *Nature* 2014, 513(7517):237-241.

158. Erdem HB, Ceylan AC, Sahin I, Sever-Erdem Z, Citli S, Tatar A: Mitochondrial DNA copy number alterations in familial mediterranean fever patients. *Bratisl Lek Listy* 2018, **119**(7):425-428.
159. Alpayci M, Bozan N, Erdem S, Gunes M, Erden M: The possible underlying pathophysiological mechanisms for development of multiple sclerosis in familial Mediterranean fever. *Med Hypotheses* 2012, **78**(6):717-720.
160. Kimura T, Jain A, Choi SW, Mandell MA, Schroder K, Johansen T, Deretic V: TRIM-mediated precision autophagy targets cytoplasmic regulators of innate immunity. *J Cell Biol* 2015, **210**(6):973-989.
161. Mitroulis I, Kourtzelis I, Kambas K, Chrysanthopoulou A, Ritis K: Evidence for the involvement of mTOR inhibition and basal autophagy in familial Mediterranean fever phenotype. *Hum Immunol* 2011, **72**(2):135-138.
162. McDermott MF, Aksentijevich I, Galon J, McDermott EM, Ogunkolade BW, Centola M, Mansfield E, Gadina M, Karenko L, Pettersson T *et al*: Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. *Cell* 1999, **97**(1):133-144.
163. Bachetti T, Chiesa S, Castagnola P, Bani D, Di Zanni E, Omenetti A, D'Osualdo A, Fraldi A, Ballabio A, Ravazzolo R *et al*: Autophagy contributes to inflammation in patients with TNFR-associated periodic syndrome (TRAPS). *Ann Rheum Dis* 2013, **72**(6):1044-1052.
164. Bulua AC, Simon A, Maddipati R, Pelletier M, Park H, Kim KY, Sack MN, Kastner DL, Siegel RM: Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *J Exp Med* 2011, **208**(3):519-533.
165. Houten SM, Kuis W, Duran M, de Koning TJ, van Royen-Kerkhof A, Romeijn GJ, Frenkel J, Dorland L, de Barse MM, Huijbers WA *et al*: Mutations in MVK, encoding mevalonate kinase, cause hyperimmunoglobulinaemia D and periodic fever syndrome. *Nat Genet* 1999, **22**(2):175-177.
166. Drenth JP, Cuisset L, Grateau G, Vasseur C, van de Velde-Visser SD, de Jong JG, Beckmann JS, van der Meer JW, Delpech M: Mutations in the gene encoding mevalonate kinase cause hyper-IgD and periodic fever syndrome. International Hyper-IgD Study Group. *Nat Genet* 1999, **22**(2):178-181.
167. Bodar EJ, van der Hilst JC, Drenth JP, van der Meer JW, Simon A: Effect of etanercept and anakinra on inflammatory attacks in the hyper-IgD syndrome: introducing a vaccination provocation model. *Neth J Med* 2005, **63**(7):260-264.

168. Park YH, Wood G, Kastner DL, Chae JJ: **Pyrin inflammasome activation and RhoA signaling in the autoinflammatory diseases FMF and HIDS**. *Nat Immunol* 2016, **17**(8):914-921.
169. Celsi F, Piscianz E, Romano M, Crovella S: **Knockdown of MVK does not lead to changes in NALP3 expression or activation**. *J Inflamm (Lond)* 2015, **12**.
170. Tricarico PM, Crovella S, Celsi F: **Mevalonate Pathway Blockade, Mitochondrial Dysfunction and Autophagy: A Possible Link**. *Int J Mol Sci* 2015, **16**(7):16067-16084.
171. van der Burgh R, Nijhuis L, Pervolaraki K, Compeer EB, Jongeneel LH, van Gijn M, Coffer PJ, Murphy MP, Mastroberardino PG, Frenkel J *et al*: **Defects in mitochondrial clearance predispose human monocytes to interleukin-1beta hypersecretion**. *J Biol Chem* 2014, **289**(8):5000-5012.
172. van der Burgh R, Pervolaraki K, Turkenburg M, Waterham HR, Frenkel J, Boes M: **Unprenylated RhoA contributes to IL-1beta hypersecretion in mevalonate kinase deficiency model through stimulation of Rac1 activity**. *J Biol Chem* 2014, **289**(40):27757-27765.
173. Dodé C, Le Dû N, Cuisset L, Letourneur F, Berthelot JM, Vaudour G, Meyrier A, Watts RA, Scott DGI, Nicholls A *et al*: **New Mutations of CIAS1 That Are Responsible for Muckle-Wells Syndrome and Familial Cold Urticaria: A Novel Mutation Underlies Both Syndromes**. *Am J Hum Genet* 2002, **70**(6):1498-1506.
174. Aganna E, Martinon F, Hawkins PN, Ross JB, Swan DC, Booth DR, Lachmann HJ, Bybee A, Gaudet R, Woo P *et al*: **Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis**. *Arthritis Rheum* 2002, **46**(9):2445-2452.
175. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD: **Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome**. *Nat Genet* 2001, **29**(3):301-305.
176. Feldmann J, Prieur AM, Quartier P, Berquin P, Certain S, Cortis E, Teillac-Hamel D, Fischer A, de Saint Basile G: **Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes**. *Am J Hum Genet* 2002, **71**(1):198-203.
177. Aksentijevich I, Nowak M, Mallah M, Chae JJ, Watford WT, Hofmann SR, Stein L, Russo R, Goldsmith D, Dent P *et al*: **De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new**

member of the expanding family of pyrin-associated autoinflammatory diseases. *Arthritis Rheum* 2002, **46**(12):3340-3348.
178. Neven B, Callebaut I, Prieur AM, Feldmann J, Bodemer C, Lepore L, Derfalvi B, Benjaponpitak S, Vesely R, Sauvain MJ *et al*: **Molecular basis of the spectral expression of CIAS1 mutations associated with phagocytic cell-mediated autoinflammatory disorders CINCA/NOMID, MWS, and FCU**. *Blood* 2004, **103**(7):2809-2815.
179. Albrecht M, Domingues FS, Schreiber S, Lengauer T: **Structural localization of disease-associated sequence variations in the NACHT and LRR domains of PYPAF1 and NOD2**. *FEBS Lett* 2003, **554**(3):520-528.
180. Carta S, Tassi S, Delfino L, Omenetti A, Raffa S, Torrisi MR, Martini A, Gattorno M, Rubartelli A: **Deficient production of IL-1 receptor antagonist and IL-6 coupled to oxidative stress in cryopyrin-associated periodic syndrome monocytes**. *Ann Rheum Dis* 2012, **71**(9):1577-1581.
181. Shinar Y, Tohami T, Livneh A, Schiby G, Hirshberg A, Nagar M, Goldstein I, Cohen R, Kukuy O, Shubman O *et al*: **Acquired familial Mediterranean fever associated with a somatic MEFV mutation in a patient with JAK2 associated post-polycythemia myelofibrosis**. *Orphanet J Rare Dis* 2015, **10**:86.
182. Rowczenio DM, Trojer H, Omoyinmi E, Arostegui JI, Arakelov G, Mensa-Vilaro A, Baginska A, Silva Pilorz C, Wang G, Lane T *et al*: **Brief Report: Association of Tumor Necrosis Factor Receptor-Associated Periodic Syndrome With Gonosomal Mosaicism of a Novel 24-Nucleotide TNFRSF1A Deletion**. *Arthritis Rheumatol* 2016, **68**(8):2044-2049.
183. Saito M, Fujisawa A, Nishikomori R, Kambe N, Nakata-Hizume M, Yoshimoto M, Ohmori K, Okafuji I, Yoshioka T, Kusunoki T *et al*: **Somatic mosaicism of CIAS1 in a patient with chronic infantile neurologic, cutaneous, articular syndrome**. *Arthritis Rheum* 2005, **52**(11):3579-3585.
184. Chen G, Sun L, Kato T, Okuda K, Martino MB, Abzhanova A, Lin JM, Gilmore RC, Batson BD, O'Neal YK *et al*: **IL-1beta dominates the promucin secretory cytokine profile in cystic fibrosis**. *J Clin Invest* 2019, **129**(10):4433-4450.
185. McElvaney OJ, Zaslona Z, Becker-Flegler K, Palsson-McDermott EM, Boland F, Gunaratnam C, Gulbins E, O'Neill LA, Reeves EP, McElvaney NG: **Specific Inhibition of the NLRP3 Inflammasome as an Antiinflammatory Strategy in Cystic Fibrosis**. *Am J Respir Crit Care Med* 2019, **200**(11):1381-1391.
186. Glick D, Barth S, Macleod KF: **Autophagy: cellular and molecular mechanisms**. *J Pathol* 2010, **221**(1):3-12.

187. Saftig P, Beertsen W, Eskelinen EL: LAMP-2: a control step for phagosome and autophagosome maturation. *Autophagy* 2008, 4(4):510-512.
188. Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y: Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol* 2009, 10(7):458-467.
189. Ganley IG, Lam du H, Wang J, Ding X, Chen S, Jiang X: ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem* 2009, 284(18):12297-12305.
190. Ravanan P, Srikumar IF, Talwar P: Autophagy: The spotlight for cellular stress responses. *Life Sci* 2017, 188:53-67.
191. Russell RC, Tian Y, Yuan H, Park HW, Chang YY, Kim J, Kim H, Neufeld TP, Dillin A, Guan KL: ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat Cell Biol* 2013, 15(7):741-750.
192. Yla-Anttila P, Vihinen H, Jokitalo E, Eskelinen EL: 3D tomography reveals connections between the phagophore and endoplasmic reticulum. *Autophagy* 2009, 5(8):1180-1185.
193. Puri C, Renna M, Bento C, Moreau K, Rubinsztein D: Diverse Autophagosome Membrane Sources Coalesce in Recycling Endosomes. *Cell* 2013, 154(6):1285-1299.
194. Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J: Mitochondria supply membranes for autophagosome biogenesis during starvation. *Cell* 2010, 141(4):656-667.
195. Ge L, Melville D, Zhang M, Schekman R: The ER-Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis. *Elife* 2013, 2:e00947.
196. Otomo C, Metlagel Z, Takaesu G, Otomo T: Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy. *Nat Struct Mol Biol* 2013, 20(1):59-66.
197. Kimura S, Noda T, Yoshimori T: Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes. *Cell Struct Funct* 2008, 33(1):109-122.
198. Lawrence RE, Zoncu R: The lysosome as a cellular centre for signalling, metabolism and quality control. *Nat Cell Biol* 2019, 21(2):133-142.
199. Saleeb RS, Kavanagh DM, Dun AR, Dalgarno PA, Duncan RR: A VPS33A-binding motif on syntaxin 17 controls autophagy completion in mammalian cells. *J Biol Chem* 2019, 294(11):4188-4201.
200. Itakura E, Kishi-Itakura C, Mizushima N: The hairpin-type tail-anchored SNARE syntaxin 17 targets to autophagosomes for fusion with endosomes/lysosomes. *Cell* 2012, 151(6):1256-1269.

201. McEwan DG, Popovic D, Gubas A, Terawaki S, Suzuki H, Stadel D, Coxon FP, Miranda de Stegmann D, Bhogaraju S, Maddi K *et al*: PLEKHM1 regulates autophagosome-lysosome fusion through HOPS complex and LC3/GABARAP proteins. *Mol Cell* 2015, 57(1):39-54.
202. Jiang P, Nishimura T, Sakamaki Y, Itakura E, Hatta T, Natsume T, Mizushima N: The HOPS complex mediates autophagosome-lysosome fusion through interaction with syntaxin 17. *Mol Biol Cell* 2014, 25(8):1327-1337.
203. Wang Z, Miao G, Xue X, Guo X, Yuan C, Zhang G, Chen Y, Feng D, Hu J, Zhang H: The Vici Syndrome Protein EPG5 Is a Rab7 Effector that Determines the Fusion Specificity of Autophagosomes with Late Endosomes/Lysosomes. *Mol Cell* 2016, 63(5):781-795.
204. Schroder BA, Wrocklage C, Hasilik A, Saftig P: The proteome of lysosomes. *Proteomics* 2010, 10(22):4053-4076.
205. Xiong J, Zhu MX: Regulation of lysosomal ion homeostasis by channels and transporters. *Sci China Life Sci* 2016, 59(8):777-791.
206. Xu H, Ren D: Lysosomal physiology. *Annu Rev Physiol* 2015, 77:57-80.
207. Mitroulis I, Kourtzelis I, Kambas K, Rafail S, Chrysanthopoulou A, Speletas M, Ritis K: Regulation of the autophagic machinery in human neutrophils. *Eur J Immunol* 2010, 40(5):1461-1472.
208. Bhattacharya A, Wei Q, Shin JN, Abdel Fattah E, Bonilla DL, Xiang Q, Eissa NT: Autophagy Is Required for Neutrophil-Mediated Inflammation. *Cell Rep* 2015, 12(11):1731-1739.
209. Jacquel A, Obba S, Boyer L, Dufies M, Robert G, Gounon P, Lemichez E, Luciano F, Solary E, Auberger P: Autophagy is required for CSF-1-induced macrophagic differentiation and acquisition of phagocytic functions. *Blood* 2012, 119(19):4527-4531.
210. Zhang Y, Morgan MJ, Chen K, Choksi S, Liu ZG: Induction of autophagy is essential for monocyte-macrophage differentiation. *Blood* 2012, 119(12):2895-2905.
211. Obba S, Hizir Z, Boyer L, Selimoglu-Buet D, Pfeifer A, Michel G, Hamouda MA, Goncalves D, Cerezo M, Marchetti S *et al*: The PRKAA1/AMPKalpha1 pathway triggers autophagy during CSF1-induced human monocyte differentiation and is a potential target in CMML. *Autophagy* 2015, 11(7):1114-1129.
212. Ghislat G, Lawrence T: Autophagy in dendritic cells. *Cell Mol Immunol* 2018, 15(11):944-952.
213. Pei B, Zhao M, Miller BC, Vela JL, Bruinsma MW, Virgin HW, Kronenberg M: Invariant NKT cells require autophagy to coordinate proliferation and

survival signals during differentiation. *J Immunol* 2015, **194**(12):5872-5884.
214. Stephenson LM, Miller BC, Ng A, Eisenberg J, Zhao Z, Cadwell K, Graham DB, Mizushima NN, Xavier R, Virgin HW *et al*: **Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes**. *Autophagy* 2009, **5**(5):625-635.
215. Miller BC, Zhao Z, Stephenson LM, Cadwell K, Pua HH, Lee HK, Mizushima NN, Iwasaki A, He YW, Swat W *et al*: **The autophagy gene ATG5 plays an essential role in B lymphocyte development**. *Autophagy* 2008, **4**(3):309-314.
216. Agyemang AF, Harrison SR, Siegel RM, McDermott MF: **Protein misfolding and dysregulated protein homeostasis in autoinflammatory diseases and beyond**. *Semin Immunopathol* 2015, **37**(4):335-347.
217. Rodgers MA, Bowman JW, Liang Q, Jung JU: **Regulation where autophagy intersects the inflammasome**. *Antioxid Redox Signal* 2014, **20**(3):495-506.
218. Shi CS, Shenderov K, Huang NN, Kabat J, Abu-Asab M, Fitzgerald KA, Sher A, Kehrl JH: **Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction**. *Nat Immunol* 2012, **13**(3):255-263.
219. Harris J, Hartman M, Roche C, Zeng SG, O'Shea A, Sharp FA, Lambe EM, Creagh EM, Golenbock DT, Tschopp J *et al*: **Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation**. *J Biol Chem* 2011, **286**(11):9587-9597.
220. Jabir MS, Hopkins L, Ritchie ND, Ullah I, Bayes HK, Li D, Tourlomousis P, Lupton A, Puleston D, Simon AK *et al*: **Mitochondrial damage contributes to Pseudomonas aeruginosa activation of the inflammasome and is downregulated by autophagy**. *Autophagy* 2015, **11**(1):166-182.
221. Wu J, Li X, Zhu G, Zhang Y, He M, Zhang J: **The role of Resveratrol-induced mitophagy/autophagy in peritoneal mesothelial cells inflammatory injury via NLRP3 inflammasome activation triggered by mitochondrial ROS**. *Exp Cell Res* 2016, **341**(1):42-53.
222. Abdelaziz DH, Khalil H, Cormet-Boyaka E, Amer AO: **The cooperation between the autophagy machinery and the inflammasome to implement an appropriate innate immune response: do they regulate each other?** *Immunol Rev* 2015, **265**(1):194-204.
223. Liu L, Yang M, Kang R, Dai Y, Yu Y, Gao F, Wang H, Sun X, Li X, Li J *et al*: **HMGB1-DNA complex-induced autophagy limits AIM2 inflammasome activation through RAGE**. *Biochem Biophys Res Commun* 2014, **450**(1):851-856.

224. Cho MH, Cho K, Kang HJ, Jeon EY, Kim HS, Kwon HJ, Kim HM, Kim DH, Yoon SY: Autophagy in microglia degrades extracellular beta-amyloid fibrils and regulates the NLRP3 inflammasome. *Autophagy* 2014, 10(10):1761-1775.
225. Shi H, Zhang Z, Wang X, Li R, Hou W, Bi W, Zhang X: Inhibition of autophagy induces IL-1beta release from ARPE-19 cells via ROS mediated NLRP3 inflammasome activation under high glucose stress. *Biochem Biophys Res Commun* 2015, 463(4):1071-1076.
226. Aflaki E, Moaven N, Borger DK, Lopez G, Westbroek W, Chae JJ, Marugan J, Patnaik S, Maniwang E, Gonzalez AN *et al*: Lysosomal storage and impaired autophagy lead to inflammasome activation in Gaucher macrophages. *Aging Cell* 2016, 15(1):77-88.
227. Kimmey JM, Huynh JP, Weiss LA, Park S, Kambal A, Debnath J, Virgin HW, Stallings CL: Unique role for ATG5 in neutrophil-mediated immunopathology during M. tuberculosis infection. *Nature* 2015, 528(7583):565-569.
228. Pu Q, Gan C, Li R, Li Y, Tan S, Li X, Wei Y, Lan L, Deng X, Liang H *et al*: Atg7 Deficiency Intensifies Inflammasome Activation and Pyroptosis in Pseudomonas Sepsis. *J Immunol* 2017, 198(8):3205-3213.
229. Bechelli J, Vergara L, Smalley C, Buzhdygan TP, Bender S, Zhang W, Liu Y, Popov VL, Wang J, Garg N *et al*: Atg5 Supports Rickettsia australis Infection in Macrophages In Vitro and In Vivo. *Infect Immun* 2019, 87(1).
230. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, Omori H, Noda T, Yamamoto N, Komatsu M *et al*: Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* 2008, 456(7219):264-268.
231. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, Englert JA, Rabinovitch M, Cernadas M, Kim HP *et al*: Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* 2011, 12(3):222-230.
232. Iula L, Keitelman IA, Sabbione F, Fuentes F, Guzman M, Galletti JG, Gerber PP, Ostrowski M, Geffner JR, Jancic CC *et al*: Autophagy Mediates Interleukin-1β Secretion in Human Neutrophils. *Front Immunol* 2018, 9.
233. Mercalli A, Calavita I, Dugnani E, Citro A, Cantarelli E, Nano R, Melzi R, Maffi P, Secchi A, Sordi V *et al*: Rapamycin unbalances the polarization of human macrophages to M1. *Immunology* 2013, 140(2):179-190.
234. McBride HM, Neuspiel M, Wasiak S: Mitochondria: more than just a powerhouse. *Curr Biol* 2006, 16(14):R551-560.

235. Tait SWG, Green DR: **Mitochondria and cell signalling**. *J Cell Sci* 2012, **125**(4):807-815.
236. Mills EL, Kelly B, O'Neill LAJ: **Mitochondria are the powerhouses of immunity**. *Nat Immunol* 2017, **18**(5):488-498.
237. Westermann B: **Mitochondrial fusion and fission in cell life and death**. *Nat Rev Mol Cell Biol* 2010, **11**(12):872-884.
238. Sun N, Youle RJ, Finkel T: **The Mitochondrial Basis of Aging**. *Mol Cell* 2016, **61**(5):654-666.
239. Cho DH, Kim YS, Jo DS, Choe SK, Jo EK: **Pexophagy: Molecular Mechanisms and Implications for Health and Diseases**. *Mol Cells* 2018, **41**(1):55-64.
240. Grumati P, Dikic I, Stolz A: **ER-phagy at a glance**. *J Cell Sci* 2018, **131**(17).
241. Cebollero E, Reggiori F, Kraft C: **Reticulophagy and Ribophagy: Regulated Degradation of Protein Production Factories**. *Int J Cell Biol* 2012, **2012**.
242. Kounakis K, Chaniotakis M, Markaki M, Tavernarakis N: **Emerging Roles of Lipophagy in Health and Disease**. *Front Cell Dev Biol* 2019, **7**.
243. Kwon DH, Song HK: **A Structural View of Xenophagy, a Battle between Host and Microbes**. *Mol Cells* 2018, **41**(1):27-34.
244. Wei H, Liu L, Chen Q: **Selective removal of mitochondria via mitophagy: distinct pathways for different mitochondrial stresses**. *Biochim Biophys Acta* 2015, **1853**(10 Pt B):2784-2790.
245. Jin SM, Lazarou M, Wang C, Kane LA, Narendra DP, Youle RJ: **Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL**. *J Cell Biol* 2010, **191**(5):933-942.
246. Kondapalli C, Kazlauskaite A, Zhang N, Woodroof HI, Campbell DG, Gourlay R, Burchell L, Walden H, Macartney TJ, Deak M *et al*: **PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65**. *Open Biol* 2012, **2**(5):120080.
247. Kazlauskaite A, Kondapalli C, Gourlay R, Campbell DG, Ritorto MS, Hofmann K, Alessi DR, Knebel A, Trost M, Muqit MM: **Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser65**. *Biochem J* 2014, **460**(1):127-139.
248. Zhang C, Lee S, Peng Y, Bunker E, Giaime E, Shen J, Zhou Z, Liu X: **PINK1 triggers autocatalytic activation of Parkin to specify cell fate decisions**. *Curr Biol* 2014, **24**(16):1854-1865.
249. Gegg ME, Cooper JM, Chau KY, Rojo M, Schapira AH, Taanman JW: **Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy**. *Hum Mol Genet* 2010, **19**(24):4861-4870.

250. Chen Y, Dorn GW, 2nd: PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. *Science* 2013, 340(6131):471-475.
251. Lazarou M: Keeping the immune system in check: a role for mitophagy. *Immunol Cell Biol* 2015, 93(1):3-10.
252. Fiesel FC, Hudec R, Springer W: Non-radioactive in vitro PINK1 Kinase Assays Using Ubiquitin or Parkin as Substrate. *Bio Protoc* 2016, 6(19).
253. Kirkin V, Lamark T, Sou YS, Bjorkoy G, Nunn JL, Bruun JA, Shvets E, McEwan DG, Clausen TH, Wild P *et al*: A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Mol Cell* 2009, 33(4):505-516.
254. von Muhlinen N, Thurston T, Ryzhakov G, Bloor S, Randow F: NDP52, a novel autophagy receptor for ubiquitin-decorated cytosolic bacteria. *Autophagy* 2010, 6(2):288-289.
255. Whang MI, Tavares RM, Benjamin DI, Kattah MG, Advincula R, Nomura DK, Debnath J, Malynn BA, Ma A: The Ubiquitin Binding Protein TAX1BP1 Mediates Autophagasome Induction and the Metabolic Transition of Activated T Cells. *Immunity* 2017, 46(3):405-420.
256. Mori F, Tanji K, Odagiri S, Toyoshima Y, Yoshida M, Kakita A, Takahashi H, Wakabayashi K: Autophagy-related proteins (p62, NBR1 and LC3) in intranuclear inclusions in neurodegenerative diseases. *Neurosci Lett* 2012, 522(2):134-138.
257. Lazarou M, Sliter DA, Kane LA, Sarraf SA, Wang C, Burman JL, Sideris DP, Fogel AI, Youle RJ: The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* 2015, 524(7565):309-314.
258. Wong YC, Holzbaur EL: Optineurin is an autophagy receptor for damaged mitochondria in parkin-mediated mitophagy that is disrupted by an ALS-linked mutation. *Proc Natl Acad Sci U S A* 2014, 111(42):E4439-4448.
259. Moore AS, Holzbaur EL: Dynamic recruitment and activation of ALS-associated TBK1 with its target optineurin are required for efficient mitophagy. *Proc Natl Acad Sci U S A* 2016, 113(24):E3349-3358.
260. Richter B, Sliter DA, Herhaus L, Stolz A, Wang C, Beli P, Zaffagnini G, Wild P, Martens S, Wagner SA *et al*: Phosphorylation of OPTN by TBK1 enhances its binding to Ub chains and promotes selective autophagy of damaged mitochondria. *Proc Natl Acad Sci U S A* 2016, 113(15):4039-4044.
261. Liu L, Feng D, Chen G, Chen M, Zheng Q, Song P, Ma Q, Zhu C, Wang R, Qi W *et al*: Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. *Nat Cell Biol* 2012, 14(2):177-185.

262. Hanna RA, Quinsay MN, Orogo AM, Giang K, Rikka S, Gustafsson AB: Microtubule-associated protein 1 light chain 3 (LC3) interacts with Bnip3 protein to selectively remove endoplasmic reticulum and mitochondria via autophagy. *J Biol Chem* 2012, 287(23):19094-19104.
263. Schwarten M, Mohrluder J, Ma P, Stoldt M, Thielmann Y, Stangler T, Hersch N, Hoffmann B, Merkel R, Willbold D: Nix directly binds to GABARAP: a possible crosstalk between apoptosis and autophagy. *Autophagy* 2009, 5(5):690-698.
264. Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, Rogov V, Lohr F, Popovic D, Occhipinti A *et al*: Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep* 2010, 11(1):45-51.
265. Zhang J, Ney PA: Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. *Cell Death Differ* 2009, 16(7):939-946.
266. Zhu Y, Massen S, Terenzio M, Lang V, Chen-Lindner S, Eils R, Novak I, Dikic I, Hamacher-Brady A, Brady NR: Modulation of serines 17 and 24 in the LC3-interacting region of Bnip3 determines pro-survival mitophagy versus apoptosis. *J Biol Chem* 2013, 288(2):1099-1113.
267. Zhang T, Xue L, Li L, Tang C, Wan Z, Wang R, Tan J, Tan Y, Han H, Tian R *et al*: BNIP3 Protein Suppresses PINK1 Kinase Proteolytic Cleavage to Promote Mitophagy. *J Biol Chem* 2016, 291(41):21616-21629.
268. Quinsay MN, Lee Y, Rikka S, Sayen MR, Molkentin JD, Gottlieb RA, Gustafsson AB: Bnip3 mediates permeabilization of mitochondria and release of cytochrome c via a novel mechanism. *J Mol Cell Cardiol* 2010, 48(6):1146-1156.
269. Quinsay MN, Thomas RL, Lee Y, Gustafsson AB: Bnip3-mediated mitochondrial autophagy is independent of the mitochondrial permeability transition pore. *Autophagy* 2010, 6(7):855-862.
270. Chen G, Han Z, Feng D, Chen Y, Chen L, Wu H, Huang L, Zhou C, Cai X, Fu C *et al*: A regulatory signaling loop comprising the PGAM5 phosphatase and CK2 controls receptor-mediated mitophagy. *Mol Cell* 2014, 54(3):362-377.
271. Chen Z, Liu L, Cheng Q, Li Y, Wu H, Zhang W, Wang Y, Sehgal SA, Siraj S, Wang X *et al*: Mitochondrial E3 ligase MARCH5 regulates FUNDC1 to fine-tune hypoxic mitophagy. *EMBO Rep* 2017, 18(3):495-509.
272. Zhou H, Zhu P, Guo J, Hu N, Wang S, Li D, Hu S, Ren J, Cao F, Chen Y: Ripk3 induces mitochondrial apoptosis via inhibition of FUNDC1 mitophagy in cardiac IR injury. *Redox Biol* 2017, 13:498-507.
273. Wu W, Tian W, Hu Z, Chen G, Huang L, Li W, Zhang X, Xue P, Zhou C, Liu L *et al*: ULK1 translocates to mitochondria and phosphorylates FUNDC1 to regulate mitophagy. *EMBO Rep* 2014, 15(5):566-575.

274. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, Kundu M, Opferman JT, Cleveland JL, Miller JL *et al*: NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci U S A* 2007, 104(49):19500-19505.
275. Yuan Y, Zheng Y, Zhang X, Chen Y, Wu X, Wu J, Shen Z, Jiang L, Wang L, Yang W *et al*: BNIP3L/NIX-mediated mitophagy protects against ischemic brain injury independent of PARK2. *Autophagy* 2017, 13(10):1754-1766.
276. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL: HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res* 2001, 61(18):6669-6673.
277. Zhang J, Loyd MR, Randall MS, Waddell MB, Kriwacki RW, Ney PA: A short linear motif in BNIP3L (NIX) mediates mitochondrial clearance in reticulocytes. *Autophagy* 2012, 8(9):1325-1332.
278. Rogov VV, Suzuki H, Marinkovic M, Lang V, Kato R, Kawasaki M, Buljubasic M, Sprung M, Rogova N, Wakatsuki S *et al*: Phosphorylation of the mitochondrial autophagy receptor Nix enhances its interaction with LC3 proteins. *Sci Rep* 2017, 7(1):1131.
279. Palikaras K, Lionaki E, Tavernarakis N: Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. *Nature* 2015, 521(7553):525-528.
280. Melser S, Chatelain EH, Lavie J, Mahfouf W, Jose C, Obre E, Goorden S, Priault M, Elgersma Y, Rezvani HR *et al*: Rheb regulates mitophagy induced by mitochondrial energetic status. *Cell Metab* 2013, 17(5):719-730.
281. Wei Y, Chiang WC, Sumpter R, Jr., Mishra P, Levine B: Prohibitin 2 Is an Inner Mitochondrial Membrane Mitophagy Receptor. *Cell* 2017, 168(1-2):224-238.e210.
282. Chu CT, Ji J, Dagda RK, Jiang JF, Tyurina YY, Kapralov AA, Tyurin VA, Yanamala N, Shrivastava IH, Mohammadyani D *et al*: Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells. *Nat Cell Biol* 2013, 15(10):1197-1205.
283. Xiao Y, Zhou Y, Lu Y, Zhou K, Cai W: PHB2 interacts with LC3 and SQSTM1 is required for bile acids-induced mitophagy in cholestatic liver. *Cell Death Dis* 2018, 9(2):160.
284. Shen Z, Li Y, Gasparski AN, Abeliovich H, Greenberg ML: Cardiolipin Regulates Mitophagy through the Protein Kinase C Pathway. *J Biol Chem* 2017, 292(7):2916-2923.
285. Esteban-Martinez L, Sierra-Filardi E, McGreal RS, Salazar-Roa M, Marino G, Seco E, Durand S, Enot D, Grana O, Malumbres M *et al*: Programmed

mitophagy is essential for the glycolytic switch during cell differentiation. *Embo j* 2017, **36**(12):1688-1706.
286. Van den Bossche J, Baardman J, Otto NA, van der Velden S, Neele AE, van den Berg SM, Luque-Martin R, Chen HJ, Boshuizen MC, Ahmed M *et al*: **Mitochondrial Dysfunction Prevents Repolarization of Inflammatory Macrophages**. *Cell Rep* 2016, **17**(3):684-696.
287. Vazquez-Martin A, Van den Haute C, Cufí S, Corominas-Faja B, Cuy**** s E, Lopez-Bonet E, Rodriguez-Gallego E, Fernández-Arroyo S, Joven J, Baekelandt V *et al*: **Mitophagy-driven mitochondrial rejuvenation regulates stem cell fate**. *Aging (Albany NY)* 2016, **8**(7):1330-1349.
288. Hu C, Fan L, Cen P, Chen E, Jiang Z, Li L: **Energy Metabolism Plays a Critical Role in Stem Cell Maintenance and Differentiation**. *Int J Mol Sci* 2016, **17**(2).
289. Shapiro BL, Feigal RJ, Lam LF: **Mitrochondrial NADH dehydrogenase in cystic fibrosis**. *Proc Natl Acad Sci U S A* 1979, **76**(6):2979-2983.
290. Shapiro BL, Lam LF, Feigal RJ: **Mitochondrial NADH dehydrogenase in cystic fibrosis: enzyme kinetics in cultured fibroblasts**. *Am J Hum Genet* 1982, **34**(6):846-852.
291. Feigal RJ, Tomczyk MS, Shapiro BL: **The calcium abnormality in cystic fibrosis mitochondria: relative role of respiration and ATP hydrolysis**. *Life Sci* 1982, **30**(1):93-98.
292. Antigny F, Girardin N, Raveau D, Frieden M, Becq F, Vandebrouck C: **Dysfunction of mitochondria Ca2+ uptake in cystic fibrosis airway epithelial cells**. *Mitochondrion* 2009, **9**(4):232-241.
293. Stutts MJ, Knowles MR, Gatzy JT, Boucher RC: **Oxygen consumption and ouabain binding sites in cystic fibrosis nasal epithelium**. *Pediatr Res* 1986, **20**(12):1316-1320.
294. Turrens JF, Freeman BA, Levitt JG, Crapo JD: **The effect of hyperoxia on superoxide production by lung submitochondrial particles**. *Arch Biochem Biophys* 1982, **217**(2):401-410.
295. Picci L, Brentagni L, Mastella G, Scarso E, Pizzochero P, Mattiazzo P, Chiandetti L, Anglani F, Zacchello F: **2D-electrophoresis of mitochondrial proteins from cystic fibrosis patients**. *Adv Exp Med Biol* 1991, **290**:379-381.
296. Bardon A, Ceder O, Kollberg H: **Increased activity of four glycolytic enzymes in cultured fibroblasts from cystic fibrosis patients**. *Res Commun Chem Pathol Pharmacol* 1986, **51**(3):405-408.
297. de Meer K, Jeneson JA, Gulmans VA, van der Laag J, Berger R: **Efficiency of oxidative work performance of skeletal muscle in patients with cystic fibrosis**. *Thorax* 1995, **50**(9):980-983.

298. Taminelli GL, Sotomayor V, Valdivieso AG, Teiber ML, Marin MC, Santa-Coloma TA: **CISD1 codifies a mitochondrial protein upregulated by the CFTR channel**. *Biochem Biophys Res Commun* 2008, **365**(4):856-862.
299. Valdivieso AG, Marcucci F, Taminelli G, Guerrico AG, Alvarez S, Teiber ML, Dankert MA, Santa-Coloma TA: **The expression of the mitochondrial gene MT-ND4 is downregulated in cystic fibrosis**. *Biochem Biophys Res Commun* 2007, **356**(3):805-809.
300. Valdivieso AG, Clauzure M, Marín MC, Taminelli GL, Massip Copiz MM, Sánchez F, Schulman G, Teiber ML, Santa-Coloma TA: **The Mitochondrial Complex I Activity Is Reduced in Cells with Impaired Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Function**. *PLoS One* 2012, **7**(11).
301. Kelly M, Trudel S, Brouillard F, Bouillaud F, Colas J, Nguyen-Khoa T, Ollero M, Edelman A, Fritsch J: **Cystic fibrosis transmembrane regulator inhibitors CFTR(inh)-172 and GlyH-101 target mitochondrial functions, independently of chloride channel inhibition**. *J Pharmacol Exp Ther* 2010, **333**(1):60-69.
302. Cleeter MW, Cooper JM, Schapira AH: **Irreversible inhibition of mitochondrial complex I by 1-methyl-4-phenylpyridinium: evidence for free radical involvement**. *J Neurochem* 1992, **58**(2):786-789.
303. Riquelme SA, Lozano C, Moustafa AM, Liimatta K, Tomlinson KL, Britto C, Khanal S, Gill SK, Narechania A, Azcona-Gutiérrez JM *et al*: **CFTR-PTEN–dependent mitochondrial metabolic dysfunction promotes Pseudomonas aeruginosa airway infection**. *Sci Transl Med* 2019, **11**(499).
304. Velsor LW, Kariya C, Kachadourian R, Day BJ: **Mitochondrial oxidative stress in the lungs of cystic fibrosis transmembrane conductance regulator protein mutant mice**. *Am J Respir Cell Mol Biol* 2006, **35**(5):579-586.
305. Kelly-Aubert M, Trudel S, Fritsch J, Nguyen-Khoa T, Baudouin-Legros M, Moriceau S, Jeanson L, Djouadi F, Matar C, Conti M *et al*: **GSH monoethyl ester rescues mitochondrial defects in cystic fibrosis models**. *Hum Mol Genet* 2011, **20**(14):2745-2759.
306. Cantin AM: **Potential for antioxidant therapy of cystic fibrosis**. *Curr Opin Pulm Med* 2004, **10**(6):531-536.
307. Anderson MF, Nilsson M, Sims NR: **Glutathione monoethylester prevents mitochondrial glutathione depletion during focal cerebral ischemia**. *Neurochem Int* 2004, **44**(3):153-159.
308. Anderson ME, Powrie F, Puri RN, Meister A: **Glutathione monoethyl ester: preparation, uptake by tissues, and conversion to glutathione**. *Arch Biochem Biophys* 1985, **239**(2):538-548.

309. Atlante A, Favia M, Bobba A, Guerra L, Casavola V, Reshkin SJ: Characterization of mitochondrial function in cells with impaired cystic fibrosis transmembrane conductance regulator (CFTR) function. *J Bioenerg Biomembr* 2016, **48**(3):197-210.
310. Wetmore DR, Joseloff E, Pilewski J, Lee DP, Lawton KA, Mitchell MW, Milburn MV, Ryals JA, Guo L: Metabolomic Profiling Reveals Biochemical Pathways and Biomarkers Associated with Pathogenesis in Cystic Fibrosis Cells*. *J Biol Chem* 2010, **285**(40):30516-30522.
311. Favia M, de Bari L, Lassandro R, Atlante A: Modulation of glucose-related metabolic pathways controls glucose level in airway surface liquid and fight oxidative stress in cystic fibrosis cells. *J Bioenerg Biomembr* 2019, **51**(3):203-218.
312. Tschopp J: Mitochondria: Sovereign of inflammation? *Eur J Immunol* 2011, **41**(5):1196-1202.
313. Rock KL, Latz E, Ontiveros F, Kono H: The sterile inflammatory response. *Annu Rev Immunol* 2010, **28**:321-342.
314. Rigante D, Vitale A, Lucherini OM, Cantarini L: The hereditary autoinflammatory disorders uncovered. *Autoimmun Rev* 2014, **13**(9):892-900.
315. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, Olivier AK, Sadler JJ, Knepper-Adrian V, Han R, Qiao L *et al*: Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 2013, **39**(2):311-323.
316. Park S, Juliana C, Hong S, Datta P, Hwang I, Fernandes-Alnemri T, Yu JW, Alnemri ES: The mitochondrial antiviral protein MAVS associates with NLRP3 and regulates its inflammasome activity. *J Immunol* 2013, **191**(8):4358-4366.
317. Bhatta A, Atianand M, Jiang Z, Crabtree J, Blin J, Fitzgerald KA: A Mitochondrial Micropeptide Is Required for Activation of the Nlrp3 Inflammasome. *J Immunol* 2020, **204**(2):428-437.
318. Murakami T, Ockinger J, Yu J, Byles V, McColl A, Hofer AM, Horng T: Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proc Natl Acad Sci U S A* 2012, **109**(28):11282-11287.
319. Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, Germain RN, Kastner DL, Chae JJ: The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca2+ and cAMP. *Nature* 2012, **492**(7427):123-127.
320. Adinolfi E, Callegari MG, Ferrari D, Bolognesi C, Minelli M, Wieckowski MR, Pinton P, Rizzuto R, Di Virgilio F: Basal activation of the P2X7 ATP receptor elevates mitochondrial calcium and potential, increases cellular

ATP levels, and promotes serum-independent growth. *Mol Biol Cell* 2005, 16(7):3260-3272.
321. Jouaville LS, Pinton P, Bastianutto C, Rutter GA, Rizzuto R: Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming. *Proc Natl Acad Sci U S A* 1999, 96(24):13807-13812.
322. Gunter TE, Pfeiffer DR: Mechanisms by which mitochondria transport calcium. *Am J Physiol* 1990, 258(5 Pt 1):C755-786.
323. Allbritton NL, Meyer T, Stryer L: Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science* 1992, 258(5089):1812-1815.
324. Simmen T, Lynes EM, Gesson K, Thomas G: Oxidative protein folding in the endoplasmic reticulum: tight links to the mitochondria-associated membrane (MAM). *Biochim Biophys Acta* 2010, 1798(8):1465-1473.
325. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E: Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 2008, 9(8):847-856.
326. Shi Y, Mucsi AD, Ng G: Monosodium urate crystals in inflammation and immunity. *Immunol Rev* 2010, 233(1):203-217.
327. Zhou R, Yazdi AS, Menu P, Tschopp J: A role for mitochondria in NLRP3 inflammasome activation. *Nature* 2011, 469(7329):221-225.
328. Kahns S, Kalai M, Jakobsen LD, Clark BF, Vandenabeele P, Jensen PH: Caspase-1 and caspase-8 cleave and inactivate cellular parkin. *J Biol Chem* 2003, 278(26):23376-23380.
329. Yu J, Nagasu H, Murakami T, Hoang H, Broderick L, Hoffman HM, Horng T: Inflammasome activation leads to Caspase-1-dependent mitochondrial damage and block of mitophagy. *Proc Natl Acad Sci U S A* 2014, 111(43):15514-15519.
330. Abdulrahman BA, Khweek AA, Akhter A, Caution K, Kotrange S, Abdelaziz DH, Newland C, Rosales-Reyes R, Kopp B, McCoy K *et al*: Autophagy stimulation by rapamycin suppresses lung inflammation and infection by Burkholderia cenocepacia in a model of cystic fibrosis. *Autophagy* 2011, 7(11):1359-1370.
331. Luciani A, Villella VR, Vasaturo A, Giardino I, Raia V, Pettoello-Mantovani M, D'Apolito M, Guido S, Leal T, Quaratino S *et al*: SUMOylation of tissue transglutaminase as link between oxidative stress and inflammation. *J Immunol* 2009, 183(4):2775-2784.
332. Nurminskaya MV, Belkin AM: Cellular functions of tissue transglutaminase. *Int Rev Cell Mol Biol* 2012, 294:1-97.
333. Johnston JA, Ward CL, Kopito RR: Aggresomes: a cellular response to misfolded proteins. *J Cell Biol* 1998, 143(7):1883-1898.

334. Abdulrahman BA, Khweek AA, Akhter A, Caution K, Tazi M, Hassan H, Zhang Y, Rowland PD, Malhotra S, Aeffner F *et al*: Depletion of the ubiquitin-binding adaptor molecule SQSTM1/p62 from macrophages harboring cftr DeltaF508 mutation improves the delivery of Burkholderia cenocepacia to the autophagic machinery. *J Biol Chem* 2013, 288(3):2049-2058.
335. Bajmoczi M, Gadjeva M, Alper SL, Pier GB, Golan DE: Cystic fibrosis transmembrane conductance regulator and caveolin-1 regulate epithelial cell internalization of Pseudomonas aeruginosa. *Am J Physiol Cell Physiol* 2009, 297(2):C263-277.
336. Deriy LV, Gomez EA, Zhang G, Beacham DW, Hopson JA, Gallan AJ, Shevchenko PD, Bindokas VP, Nelson DJ: Disease-causing mutations in the cystic fibrosis transmembrane conductance regulator determine the functional responses of alveolar macrophages. *J Biol Chem* 2009, 284(51):35926-35938.
337. Caution K, Pan A, Krause K, Badr A, Hamilton K, Vaidya A, Gosu H, Daily K, Estfanous S, Gavrilin MA *et al*: Methylomic correlates of autophagy activity in cystic fibrosis. *J Cyst Fibros* 2019, 18(4):491-500.
338. Kleta R, Gahl WA: Pharmacological treatment of nephropathic cystinosis with cysteamine. *Expert Opin Pharmacother* 2004, 5(11):2255-2262.
339. Tosco A, De Gregorio F, Esposito S, De Stefano D, Sana I, Ferrari E, Sepe A, Salvadori L, Buonpensiero P, Di Pasqua A *et al*: A novel treatment of cystic fibrosis acting on-target: cysteamine plus epigallocatechin gallate for the autophagy-dependent rescue of class II-mutated CFTR. *Cell Death Differ* 2016, 23(8):1380-1393.
340. Stefano DD, Villella VR, Esposito S, Tosco A, Sepe A, Gregorio FD, Salvadori L, Grassia R, Leone CA, Rosa GD *et al*: Restoration of CFTR function in patients with cystic fibrosis carrying the F508del-CFTR mutation. *Autophagy* 2014, 10(11):2053-2074.
341. Luciani A, Villella VR, Esposito S, Gavina M, Russo I, Silano M, Guido S, Pettoello-Mantovani M, Carnuccio R, Scholte B *et al*: Targeting autophagy as a novel strategy for facilitating the therapeutic action of potentiators on ΔF508 cystic fibrosis transmembrane conductance regulator. *Autophagy* 2012, 8(11):1657-1672.
342. Besouw M, Masereeuw R, van den Heuvel L, Levtchenko E: Cysteamine: an old drug with new potential. *Drug Discov Today* 2013, 18(15-16):785-792.
343. Devereux G, Fraser-Pitt D, Robertson J, Devlin E, Mercer D, O'Neil D: Cysteamine as a Future Intervention in Cystic Fibrosis Against Current and Emerging Pathogens: A Patient-based ex vivo Study Confirming its

Antimicrobial and Mucoactive Potential in Sputum. *EBioMedicine* 2015, 2(10):1507-1512.
344. Charrier C, Rodger C, Robertson J, Kowalczuk A, Shand N, Fraser-Pitt D, Mercer D, O'Neil D: Cysteamine (Lynovex(R)), a novel mucoactive antimicrobial & antibiofilm agent for the treatment of cystic fibrosis. *Orphanet J Rare Dis* 2014, 9:189.
345. Ferrari E, Monzani R, Villella VR, Esposito S, Saluzzo F, Rossin F, D'Eletto M, Tosco A, De Gregorio F, Izzo V *et al*: Cysteamine re-establishes the clearance of Pseudomonas aeruginosa by macrophages bearing the cystic fibrosis-relevant F508del-CFTR mutation. In: *Cell Death Dis.* vol. 8; 2017: e2544-.
346. Shrestha CL, Assani KD, Rinehardt H, Albastroiu F, Zhang S, Shell R, Amer AO, Schlesinger LS, Kopp BT: Cysteamine-mediated clearance of antibiotic-resistant pathogens in human cystic fibrosis macrophages. *PLoS One* 2017, 12(10).
347. Vu CB, Bridges RJ, Pena-Rasgado C, Lacerda AE, Bordwell C, Sewell A, Nichols AJ, Chandran S, Lonkar P, Picarella D *et al*: Fatty Acid Cysteamine Conjugates as Novel and Potent Autophagy Activators That Enhance the Correction of Misfolded F508del-Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). *J Med Chem* 2017, 60(1):458-473.
348. Gupta S, Hastak K, Afaq F, Ahmad N, Mukhtar H: Essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor kappa B and induction of apoptosis. *Oncogene* 2004, 23(14):2507-2522.
349. Kato T, Jr., Delhase M, Hoffmann A, Karin M: CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response. *Mol Cell* 2003, 12(4):829-839.
350. Zheng Y, Qin H, Frank SJ, Deng L, Litchfield DW, Tefferi A, Pardanani A, Lin FT, Li J, Sha B *et al*: A CK2-dependent mechanism for activation of the JAK-STAT signaling pathway. *Blood* 2011, 118(1):156-166.
351. Silva A, Yunes JA, Cardoso BA, Martins LR, Jotta PY, Abecasis M, Nowill AE, Leslie NR, Cardoso AA, Barata JT: PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest* 2008, 118(11):3762-3774.
352. Zhang S, Stoll G, Pedro J, Sica V, Sauvat A, Obrist F, Kepp O, Li Y, Maiuri L, Zamzami N *et al*: Evaluation of autophagy inducers in epithelial cells carrying the DeltaF508 mutation of the cystic fibrosis transmembrane conductance regulator CFTR. *Cell Death Dis* 2018, 9(2):191.
353. Bodas M, Vij N: Adapting Proteostasis and Autophagy for Controlling the Pathogenesis of Cystic Fibrosis Lung Disease. *Front Pharmacol* 2019, 10.

354. Villella VR, Esposito S, Bruscia EM, Maiuri MC, Raia V, Kroemer G, Maiuri L: **Targeting the Intracellular Environment in Cystic Fibrosis: Restoring Autophagy as a Novel Strategy to Circumvent the CFTR Defect**. *Front Pharmacol* 2013, **4**.
355. Blohmke CJ, Mayer ML, Tang AC, Hirschfeld AF, Fjell CD, Sze MA, Falsafi R, Wang S, Hsu K, Chilvers MA *et al*: **Atypical activation of the unfolded protein response in cystic fibrosis airway cells contributes to p38 MAPK-mediated innate immune responses**. *J Immunol* 2012, **189**(11):5467-5475.
356. Ward CL, Omura S, Kopito RR: **Degradation of CFTR by the ubiquitin-proteasome pathway**. *Cell* 1995, **83**(1):121-127.
357. Maiuri L, Luciani A, Giardino I, Raia V, Villella VR, D'Apolito M, Pettoello-Mantovani M, Guido S, Ciacci C, Cimmino M *et al*: **Tissue Transglutaminase Activation Modulates Inflammation in Cystic Fibrosis via PPARγ Down-Regulation**. 2008.
358. Teichgraber V, Ulrich M, Endlich N, Riethmuller J, Wilker B, De Oliveira-Munding CC, van Heeckeren AM, Barr ML, von Kurthy G, Schmid KW *et al*: **Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis**. *Nat Med* 2008, **14**(4):382-391.
359. Haggie PM, Verkman AS: **Unimpaired lysosomal acidification in respiratory epithelial cells in cystic fibrosis**. *J Biol Chem* 2009, **284**(12):7681-7686.
360. Liu J, Lu W, Guha S, Baltazar GC, Coffey EE, Laties AM, Rubenstein RC, Reenstra WW, Mitchell CH: **Cystic fibrosis transmembrane conductance regulator contributes to reacidification of alkalinized lysosomes in RPE cells**. *Am J Physiol Cell Physiol* 2012, **303**(2):C160-169.
361. Van Dyke RW, Root KV, Schreiber JH, Wilson JM: **Role of CFTR in lysosome acidification**. *Biochem Biophys Res Commun* 1992, **184**(1):300-305.
362. Senft D, Ronai ZA: **UPR, autophagy, and mitochondria crosstalk underlies the ER stress response**. *Trends Biochem Sci* 2015, **40**(3):141-148.
363. Takahama M, Akira S, Saitoh T: **Autophagy limits activation of the inflammasomes**. *Immunol Rev* 2018, **281**(1):62-73.
364. Tazi MF, Dakhlallah DA, Caution K, Gerber MM, Chang SW, Khalil H, Kopp BT, Ahmed AE, Krause K, Davis I *et al*: **Elevated Mirc1/Mir17-92 cluster expression negatively regulates autophagy and CFTR (cystic fibrosis transmembrane conductance regulator) function in CF macrophages**. *Autophagy* 2016, **12**(11):2026-2037.
365. Du K, Karp PH, Ackerley C, Zabner J, Keshavjee S, Cutz E, Yeger H: **Aggregates of mutant CFTR fragments in airway epithelial cells of CF lungs: new pathologic observations**. *J Cyst Fibros* 2015, **14**(2):182-193.

366. Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, Mizushima N, Iwata J, Ezaki J, Murata S *et al*: Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell* 2007, 131(6):1149-1163.
367. Zhong Z, Umemura A, Sanchez-Lopez E, Liang S, Shalapour S, Wong J, He F, Boassa D, Perkins G, Ali SR *et al*: NF-κB Restricts inflammasome activation via elimination of damaged mitochondria. *Cell* 2016, 164(5):896-910.
368. Song P, Li S, Wu H, Gao R, Rao G, Wang D, Chen Z, Ma B, Wang H, Sui N *et al*: Parkin promotes proteasomal degradation of p62: implication of selective vulnerability of neuronal cells in the pathogenesis of Parkinson's disease. *Protein Cell* 2016, 7(2):114-129.
369. Hsieh CW, Chang CY, Chen YM, Chen HH, Hung WT, Gung NR, Wey SJ, Chen DY: Impaired autophagic flux and its related inflammation in patients with adult-onset Still's disease. *Oncotarget* 2018, 9(1):110-121.
370. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G, Gelinas C *et al*: Autophagy suppresses tumorigenesis through elimination of p62. *Cell* 2009, 137(6):1062-1075.
371. Tabary O, Escotte S, Couetil JP, Hubert D, Dusser D, Puchelle E, Jacquot J: Genistein inhibits constitutive and inducible NFkappaB activation and decreases IL-8 production by human cystic fibrosis bronchial gland cells. *Am J Pathol* 1999, 155(2):473-481.
372. DiMango E, Ratner AJ, Bryan R, Tabibi S, Prince A: Activation of NF-kappaB by adherent Pseudomonas aeruginosa in normal and cystic fibrosis respiratory epithelial cells. *J Clin Invest* 1998, 101(11):2598-2605.
373. Tabary O, Escotte S, Couetil JP, Hubert D, Dusser D, Puchelle E, Jacquot J: Relationship between IkappaBalpha deficiency, NFkappaB activity and interleukin-8 production in CF human airway epithelial cells. *Pflugers Arch* 2001, 443 Suppl 1:S40-44.
374. Momtazi G, Elbanna A, Finnegan S, Héritier M, Lavery L, Mccollum K, Naranjo J, Schock B: NF-kB activation in cystic fibrosis: The dream of A20. 2019.
375. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, Springer W: PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 2010, 12(2):119-131.
376. Kageyama S, Saito T, Obata M, Koide R, Ichimura Y, Komatsu M: Negative Regulation of the Keap1-Nrf2 Pathway by a p62/Sqstm1 Splicing Variant. *Mol Cell Biol* 2018, 38(7).

377. Ichimura Y, Komatsu M: Activation of p62/SQSTM1–Keap1–Nuclear Factor Erythroid 2-Related Factor 2 Pathway in Cancer. *Front Oncol* 2018, 8.
378. Clarke AJ, Simon AK: Autophagy in the renewal, differentiation and homeostasis of immune cells. *Nat Rev Immunol* 2019, 19(3):170-183.
379. Cholon DM, Quinney NL, Fulcher ML, Esther CR, Das J, Dokholyan NV, Randell SH, Boucher RC, Gentzsch M: Potentiator Ivacaftor Abrogates Pharmacological Correction of ΔF508 CFTR in Cystic Fibrosis. *Sci Transl Med* 2014, 6(246):246ra296.
380. Zhou Y, Song K, Painter RG, Aiken M, Reiser J, Stanton BA, Nauseef WM, Wang G: Cystic fibrosis transmembrane conductance regulator recruitment to phagosomes in neutrophils. *J Innate Immun* 2013, 5(3):219-230.
381. Li C, Wu Y, Riehle A, Ma J, Kamler M, Gulbins E, Grassmé H: Staphylococcus aureus Survives in Cystic Fibrosis Macrophages, Forming a Reservoir for Chronic Pneumonia. *Infect Immun* 2017, 85(5).
382. Kelly C, Canning P, Buchanan PJ, Williams MT, Brown V, Gruenert DC, Elborn JS, Ennis M, Schock BC: Toll-like receptor 4 is not targeted to the lysosome in cystic fibrosis airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2013, 304(5):L371-382.
383. Eapen M, Tan E, Walters H, Sohal SS, Ward C: Increased expression of Lysosomal-associated membrane protein 1 (LAMP-1) found in small airway epithelium of COPD may have functional consequence and role in autophagy. 2017.
384. Dhalla NS, Temsah RM, Netticadan T: Role of oxidative stress in cardiovascular diseases. *J Hypertens* 2000, 18(6):655-673.
385. Kim GH, Kim JE, Rhie SJ, Yoon S: The Role of Oxidative Stress in Neurodegenerative Diseases. *Exp Neurobiol* 2015, 24(4):325-340.
386. McGuinness AJA, Sapey E: Oxidative Stress in COPD: Sources, Markers, and Potential Mechanisms. *J Clin Med* 2017, 6(2).
387. Galli F, Battistoni A, Gambari R, Pompella A, Bragonzi A, Pilolli F, Iuliano L, Piroddi M, Dechecchi MC, Cabrini G: Oxidative stress and antioxidant therapy in cystic fibrosis. *Biochim Biophys Acta* 2012, 1822(5):690-713.
388. AM C, G B, C O, J L, JW H: Oxidant Stress Suppresses CFTR Expression. *American journal of physiology Cell physiology* 2006, 290(1).
389. Ahmed SM, Luo L, Namani A, Wang XJ, Tang X: Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochim Biophys Acta Mol Basis Dis* 2017, 1863(2):585-597.
390. Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M: Oxidative stress sensor Keap1 functions as an adaptor for

Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol* 2004, **24**(16):7130-7139.
391. Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA: **The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase**. *Mol Cell Biol* 2004, **24**(19):8477-8486.
392. Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M: **Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex**. *Mol Cell Biol* 2004, **24**(24):10941-10953.
393. Zhang DD, Hannink M: **Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress**. *Mol Cell Biol* 2003, **23**(22):8137-8151.
394. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, Sou YS, Ueno I, Sakamoto A, Tong KI *et al*: **The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1**. *Nat Cell Biol* 2010, **12**(3):213-223.
395. Copple IM, Lister A, Obeng AD, Kitteringham NR, Jenkins RE, Layfield R, Foster BJ, Goldring CE, Park BK: **Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway**. *J Biol Chem* 2010, **285**(22):16782-16788.
396. Lau A, Wang XJ, Zhao F, Villeneuve NF, Wu T, Jiang T, Sun Z, White E, Zhang DD: **A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62**. *Mol Cell Biol* 2010, **30**(13):3275-3285.
397. Jain A, Lamark T, Sjottem E, Larsen KB, Awuh JA, Overvatn A, McMahon M, Hayes JD, Johansen T: **p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription**. *J Biol Chem* 2010, **285**(29):22576-22591.
398. Chhikara M, Wang S, Kern SJ, Ferreyra GA, Barb JJ, Munson PJ, Danner RL: **Carbon Monoxide Blocks Lipopolysaccharide-Induced Gene Expression by Interfering with Proximal TLR4 to NF-κB Signal Transduction in Human Monocytes**. *PLoS One* 2009, **4**(12).
399. Liu Y, Zhu B, Wang X, Luo L, Li P, Paty DW, Cynader MS: **Bilirubin as a potent antioxidant suppresses experimental autoimmune encephalomyelitis: implications for the role of oxidative stress in the development of multiple sclerosis**. *J Neuroimmunol* 2003, **139**(1-2):27-35.
400. Zhang F, Sun Y, Fan K, Dong X, Han N, Zhao H, Kong L: **Protective effects of heme oxygenase-1 against severe acute pancreatitis via inhibition of**

tumor necrosis factor-α and augmentation of interleukin-10. *BMC Gastroenterol* 2017, **17**.
401. Chen HG, Xie KL, Han HZ, Wang WN, Liu DQ, Wang GL, Yu YH: **Heme oxygenase-1 mediates the anti-inflammatory effect of molecular hydrogen in LPS-stimulated RAW 264.7 macrophages**. *Int J Surg* 2013, **11**(10):1060-1066.
402. Ross D, Siegel D: **Functions of NQO1 in Cellular Protection and CoQ10 Metabolism and its Potential Role as a Redox Sensitive Molecular Switch**. *Front Physiol* 2017, **8**:595.
403. Yang H, Magilnick N, Lee C, Kalmaz D, Ou X, Chan JY, Lu SC: **Nrf1 and Nrf2 Regulate Rat Glutamate-Cysteine Ligase Catalytic Subunit Transcription Indirectly via NF-κB and AP-1**. *Mol Cell Biol* 2005, **25**(14):5933-5946.
404. Yang HC, Wu YH, Yen WC, Liu HY, Hwang TL, Stern A, Chiu DTY: **The Redox Role of G6PD in Cell Growth, Cell Death, and Cancer**. *Cells* 2019, **8**(9).
405. Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H, Tanaka N, Moriguchi T, Motohashi H, Nakayama K *et al*: **Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription**. *Nat Commun* 2016, **7**:11624.
406. Osburn WO, Karim B, Dolan PM, Liu G, Yamamoto M, Huso DL, Kensler TW: **Increased colonic inflammatory injury and formation of aberrant crypt foci in Nrf2-deficient mice upon dextran sulfate treatment**. *Int J Cancer* 2007, **121**(9):1883-1891.
407. Thimmulappa RK, Scollick C, Traore K, Yates M, Trush MA, Liby KT, Sporn MB, Yamamoto M, Kensler TW, Biswal S: **Nrf2-dependent protection from LPS induced inflammatory response and mortality by CDDO-Imidazolide**. *Biochem Biophys Res Commun* 2006, **351**(4):883-889.
408. Liu X, Zhang X, Ding Y, Zhou W, Tao L, Lu P, Wang Y, Hu R: **Nuclear Factor E2-Related Factor-2 Negatively Regulates NLRP3 Inflammasome Activity by Inhibiting Reactive Oxygen Species-Induced NLRP3 Priming**. *Antioxid Redox Signal* 2017, **26**(1):28-43.
409. Zhao C, Gillette DD, Li X, Zhang Z, Wen H: **Nuclear factor E2-related factor-2 (Nrf2) is required for NLRP3 and AIM2 inflammasome activation**. *J Biol Chem* 2014, **289**(24):17020-17029.
410. Chen J, Kinter M, Shank S, Cotton C, Kelley TJ, Ziady AG: **Dysfunction of Nrf-2 in CF epithelia leads to excess intracellular H2O2 and inflammatory cytokine production**. *PLoS One* 2008, **3**(10):e3367.
411. Ziady AG, Sokolow A, Shank S, Corey D, Myers R, Plafker S, Kelley TJ: **Interaction with CREB binding protein modulates the activities of Nrf2 and NF-kappaB in cystic fibrosis airway epithelial cells**. *Am J Physiol Lung Cell Mol Physiol* 2012, **302**(11):L1221-1231.

412. Li J, Stein TD, Johnson JA: **Genetic dissection of systemic autoimmune disease in Nrf2-deficient mice**. *Physiol Genomics* 2004, **18**(3):261-272.
413. Borcherding DC, Siefert ME, Lin S, Brewington J, Sadek H, Clancy JP, Plafker SM, Ziady AG: **Clinically approved CFTR modulators rescue Nrf2 dysfunction in cystic fibrosis airway epithelia**. *J Clin Invest*, **129**(8):3448-3463.
414. O'Mealey GB, Plafker KS, Berry WL, Janknecht R, Chan JY, Plafker SM: **A PGAM5–KEAP1–Nrf2 complex is required for stress-induced mitochondrial retrograde trafficking**. *J Cell Sci* 2017, **130**(20):3467-3480.
415. Lo SC, Hannink M: **PGAM5 tethers a ternary complex containing Keap1 and Nrf2 to mitochondria**. *Exp Cell Res* 2008, **314**(8):1789-1803.
416. Ganzleben I, He GW, Gunther C, Prigge ES, Richter K, Rieker RJ, Mougiakakos D, Neurath MF, Becker C: **PGAM5 is a key driver of mitochondrial dysfunction in experimental lung fibrosis**. *Cell Mol Life Sci* 2019, **76**(23):4783-4794.
417. Ciofu O, Lykkesfeldt J: **Antioxidant supplementation for lung disease in cystic fibrosis**. *Cochrane Database Syst Rev* 2014(8):Cd007020.
418. Nash EF, Stephenson A, Ratjen F, Tullis E: **Nebulized and oral thiol derivatives for pulmonary disease in cystic fibrosis**. *Cochrane Database Syst Rev* 2009(1):Cd007168.
419. Dickerhof N, Pearson JF, Hoskin TS, Berry LJ, Turner R, Sly PD, Kettle AJ: **Oxidative stress in early cystic fibrosis lung disease is exacerbated by airway glutathione deficiency**. *Free Radic Biol Med* 2017, **113**:236-243.
420. Cloonan SM, Choi AM: **Mitochondria in lung disease**. *J Clin Invest* 2016, **126**(3):809-820.
421. Subramanian N, Natarajan K, Clatworthy MR, Wang Z, Germain RN: **The adaptor MAVS promotes NLRP3 mitochondrial localization and inflammasome activation**. *Cell* 2013, **153**(2):348-361.
422. Elliott EI, Miller AN, Banoth B, Iyer SS, Stotland A, Weiss JP, Gottlieb RA, Sutterwala FS, Cassel SL: **Mitochondrial assembly of the NLRP3 inflammasome complex is initiated at priming**. *J Immunol* 2018, **200**(9):3047-3052.
423. Cruz CM, Rinna A, Forman HJ, Ventura AL, Persechini PM, Ojcius DM: **ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages**. *J Biol Chem* 2007, **282**(5):2871-2879.
424. Gross CJ, Mishra R, Schneider KS, Medard G, Wettmarshausen J, Dittlein DC, Shi H, Gorka O, Koenig PA, Fromm S *et al*: **K(+) Efflux-Independent NLRP3 Inflammasome Activation by Small Molecules Targeting Mitochondria**. *Immunity* 2016, **45**(4):761-773.

425. O'Neill LA, Kishton RJ, Rathmell J: A guide to immunometabolism for immunologists. *Nat Rev Immunol* 2016, 16(9):553-565.
426. Everts B, Amiel E, van der Windt GJ, Freitas TC, Chott R, Yarasheski KE, Pearce EL, Pearce EJ: Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood* 2012, 120(7):1422-1431.
427. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, Tourlomousis P, Dabritz JHM, Gottlieb E, Latorre I *et al*: Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* 2016, 167(2):457-470.e413.
428. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, Logan A, Nadtochiy SM, Ord ENJ, Smith AC *et al*: Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* 2014, 515(7527):431-435.
429. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, Frezza C, Bernard NJ, Kelly B, Foley NH *et al*: Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature* 2013, 496(7444):238-242.
430. Littlewood-Evans A, Sarret S, Apfel V, Loesle P, Dawson J, Zhang J, Muller A, Tigani B, Kneuer R, Patel S *et al*: GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis. *J Exp Med* 2016, 213(9):1655-1662.
431. Mills EL, Ryan DG, Prag HA, Dikovskaya D, Menon D, Zaslona Z, Jedrychowski MP, Costa ASH, Higgins M, Hams E *et al*: Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. *Nature* 2018, 556(7699):113-117.
432. Moon JS, Hisata S, Park MA, DeNicola GM, Ryter SW, Nakahira K, Choi AMK: mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation. *Cell Rep* 2015, 12(1):102-115.
433. Cil O, Phuan PW, Gillespie AM, Lee S, Tradtrantip L, Yin J, Tse M, Zachos NC, Lin R, Donowitz M *et al*: Benzopyrimido-pyrrolo-oxazine-dione CFTR inhibitor (R)-BPO-27 for antisecretory therapy of diarrheas caused by bacterial enterotoxins. *FASEB J* 2017, 31(2):751-760.
434. Zhang Z, Qu J, Zheng C, Zhang P, Zhou W, Cui W, Mo X, Li L, Xu L, Gao J: Nrf2 antioxidant pathway suppresses Numb-mediated epithelial-mesenchymal transition during pulmonary fibrosis. *Cell Death Dis* 2018, 9(2):83.
435. Heid ME, Keyel PA, Kamga C, Shiva S, Watkins SC, Salter RD: Mitochondrial ROS induces NLRP3-dependent lysosomal damage and inflammasome activation1. *J Immunol* 2013, 191(10).

436. Surmiak MP, Hubalewska-Mazgaj M, Wawrzycka-Adamczyk K, Szczeklik W, Musiał J, Sanak M: Circulating mitochondrial DNA in serum of patients with granulomatosis with polyangiitis. *Clin Exp Immunol* 2015, 181(1):150-155.
437. Picca A, Beli R, Calvani R, Coelho-Junior HJ, Landi F, Bernabei R, Bucci C, Guerra F, Marzetti E: Older Adults with Physical Frailty and Sarcopenia Show Increased Levels of Circulating Small Extracellular Vesicles with a Specific Mitochondrial Signature. *Cells* 2020, 9(4).
438. Averna M, Bavestrello M, Cresta F, Pedrazzi M, De Tullio R, Minicucci L, Sparatore B, Salamino F, Pontremoli S, Melloni E: Abnormal activation of calpain and protein kinase Calpha promotes a constitutive release of matrix metalloproteinase 9 in peripheral blood mononuclear cells from cystic fibrosis patients. *Arch Biochem Biophys* 2016, 604:103-112.
439. Mueller C, Braag SA, Keeler A, Hodges C, Drumm M, Flotte TR: Lack of cystic fibrosis transmembrane conductance regulator in CD3+ lymphocytes leads to aberrant cytokine secretion and hyperinflammatory adaptive immune responses. *Am J Respir Cell Mol Biol* 2011, 44(6):922-929.
440. Prufer S, Weber M, Sasca D, Teschner D, Wolfel C, Stein P, Stassen M, Schild H, Radsak MP: Distinct Signaling Cascades of TREM-1, TLR and NLR in Neutrophils and Monocytic Cells. *Journal of innate immunity* 2014, 6(3).
441. Garcia-Marcos M, Fontanils U, Aguirre A, Pochet S, Dehaye JP, Marino A: Role of sodium in mitochondrial membrane depolarization induced by P2X7 receptor activation in submandibular glands. *FEBS Lett* 2005, 579(24):5407-5413.
442. Rasmussen JE, Sheridan JT, Polk W, Davies CM, Tarran R: Cigarette Smoke-induced Ca2+ Release Leads to Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Dysfunction*. *J Biol Chem* 2014, 289(11):7671-7681.
443. Balghi H, Robert R, Rappaz B, Zhang X, Wohlhuter-Haddad A, Evagelidis A, Luo Y, Goepp J, Ferraro P, Roméo P *et al*: Enhanced Ca2+ entry due to Orai1 plasma membrane insertion increases IL-8 secretion by cystic fibrosis airways. *FASEB J* 2011, 25(12):4274-4291.
444. East DA, Fagiani F, Crosby J, Georgakopoulos ND, Bertrand H, Schaap M, Fowkes A, Wells G, Campanella M: PMI: a DeltaPsim independent pharmacological regulator of mitophagy. *Chem Biol* 2014, 21(11):1585-1596.

445. Wu Y, Li JQ, Kim YJ, Wu J, Wang Q, Hao Y: In vivo and in vitro antiviral effects of berberine on influenza virus. *Chin J Integr Med* 2011, **17**(6):444-452.
446. Liu H, You L, Wu J, Zhao M, Guo R, Zhang H, Su R, Mao Q, Deng D, Hao Y: Berberine suppresses influenza virus-triggered NLRP3 inflammasome activation in macrophages by inducing mitophagy and decreasing mitochondrial ROS. *J Leukoc Biol* 2020.
447. Liang Y, Fan C, Yan X, Lu X, Jiang H, Di S, Ma Z, Feng Y, Zhang Z, Feng P *et al*: Berberine ameliorates lipopolysaccharide-induced acute lung injury via the PERK-mediated Nrf2/HO-1 signaling axis. *Phytother Res* 2019, **33**(1):130-148.
448. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, Tourlomousis P, Däbritz JHM, Gottlieb E, Latorre I *et al*: Repurposing mitochondria from ATP production to ROS generation drives a pro-inflammatory phenotype in macrophages that depends on succinate oxidation by complex II. *Cell* 2016, **167**(2):457-470 e413.
449. Peckham D, Holland E, Range S, Knox AJ: Na+/K+ ATPase in lower airway epithelium from cystic fibrosis and non-cystic-fibrosis lung. *Biochem Biophys Res Commun* 1997, **232**(2):464-468.
450. Moreno-Sánchez R, Hansford RG: Dependence of cardiac mitochondrial pyruvate dehydrogenase activity on intramitochondrial free Ca2+ concentration. *Biochem J* 1988, **256**(2):403-412.
451. Denton RM, McCormack JG, Edgell NJ: Role of calcium ions in the regulation of intramitochondrial metabolism. Effects of Na+, Mg2+ and ruthenium red on the Ca2+-stimulated oxidation of oxoglutarate and on pyruvate dehydrogenase activity in intact rat heart mitochondria. *Biochem J* 1980, **190**(1):107-117.
452. McCormack JG, Denton RM: Mitochondrial Ca2+ transport and the role of intramitochondrial Ca2+ in the regulation of energy metabolism. *Dev Neurosci* 1993, **15**(3-5):165-173.
453. Fraser-Pitt D, Mercer D, Lovie E, Robertson J, O'Neil D: Activity of Cysteamine against the Cystic Fibrosis Pathogen Burkholderia cepacia Complex. *Antimicrob Agents Chemother* 2016, **60**(10):6200-6206.
454. Zhang Y, Duan W, Owusu L, Wu D, Xin Y: Epigallocatechin-3-gallate induces the apoptosis of hepatocellular carcinoma LM6 cells but not non-cancerous liver cells. *Int J Mol Med* 2015, **35**(1):117-124.
455. Huang CY, Han Z, Li X, Xie HH, Zhu SS: Mechanism of EGCG promoting apoptosis of MCF-7 cell line in human breast cancer. *Oncol Lett* 2017, **14**(3):3623-3627.

456. Wu PP, Kuo SC, Huang WW, Yang JS, Lai KC, Chen HJ, Lin KL, Chiu YJ, Huang LJ, Chung JG: (-)-Epigallocatechin gallate induced apoptosis in human adrenal cancer NCI-H295 cells through caspase-dependent and caspase-independent pathway. *Anticancer Res* 2009, 29(4):1435-1442.
457. Khiewkamrop P, Phunsomboon P, Richert L, Pekthong D, Srisawang P: Epistructured catechins, EGCG and EC facilitate apoptosis induction through targeting de novo lipogenesis pathway in HepG2 cells. *Cancer Cell Int* 2018, 18.
458. Zhong Y, Chiou YS, Pan MH, Shahidi F: Anti-inflammatory activity of lipophilic epigallocatechin gallate (EGCG) derivatives in LPS-stimulated murine macrophages. *Food Chem* 2012, 134(2):742-748.
459. Paquay JB, Haenen GR, Stender G, Wiseman SA, Tijburg LB, Bast A: Protection against nitric oxide toxicity by tea. *J Agric Food Chem* 2000, 48(11):5768-5772.
460. Nagai K, Jiang MH, Hada J, Nagata T, Yajima Y, Yamamoto S, Nishizaki T: (-)-Epigallocatechin gallate protects against NO stress-induced neuronal damage after ischemia by acting as an anti-oxidant. *Brain Res* 2002, 956(2):319-322.
461. Hagiu A, Attin T, Schmidlin PR, Ramenzoni LL: Dose-dependent green tea effect on decrease of inflammation in human oral gingival epithelial keratinocytes: in vitro study. *Clin Oral Investig* 2019.
462. Zhong X, Liu M, Yao W, Du K, He M, Jin X, Jiao L, Ma G, Wei B, Wei M: Epigallocatechin-3-Gallate Attenuates Microglial Inflammation and Neurotoxicity by Suppressing the Activation of Canonical and Noncanonical Inflammasome via TLR4/NF-kappaB Pathway. *Mol Nutr Food Res* 2019, 63(21):e1801230.
463. Lee HE, Yang G, Park YB, Kang HC, Cho YY, Lee HS, Lee JY: Epigallocatechin-3-Gallate Prevents Acute Gout by Suppressing NLRP3 Inflammasome Activation and Mitochondrial DNA Synthesis. *Molecules* 2019, 24(11).
464. Armirotti A, Tomati V, Matthes E, Veit G, Cholon DM, Phuan PW, Braccia C, Guidone D, Gentzsch M, Lukacs GL *et al*: Bioactive Thymosin Alpha-1 Does Not Influence F508del-CFTR Maturation and Activity. *Sci Rep* 2019, 9(1):10310.
465. Mayer ML, Blohmke CJ, Falsafi R, Fjell CD, Madera L, Turvey SE, Hancock RE: Rescue of dysfunctional autophagy attenuates hyperinflammatory responses from cystic fibrosis cells. *J Immunol* 2013, 190(3):1227-1238.
466. Romani L, Oikonomou V, Moretti S, Iannitti RG, D'Adamo MC, Villella VR, Pariano M, Sforna L, Borghi M, Bellet MM *et al*: Thymosin alpha1

represents a potential potent single-molecule-based therapy for cystic fibrosis. *Nat Med* 2017, **23**(5):590-600.
467. Tomati V, Caci E, Ferrera L, Pesce E, Sondo E, Cholon DM, Quinney NL, Boyles SE, Armirotti A, Ravazzolo R *et al*: **Thymosin alpha-1 does not correct F508del-CFTR in cystic fibrosis airway epithelia**. *JCI Insight* 2018, **3**(3).
468. Matthes E, Hanrahan JW, Cantin AM: **F508del-CFTR is not corrected by thymosin alpha1**. *Nat Med* 2018, **24**(7):890-891.
469. Romani L, Stincardini C, Giovagnoli S, Paci M, Villella VR, Sforna L, Renga G, Bellet MM, Costantini C, Puccetti P *et al*: **Reply to 'F508del-CFTR is not corrected by thymosin alpha1'**. *Nat Med* 2018, **24**(7):891-893.
470. Younger JM, Chen L, Ren HY, Rosser MF, Turnbull EL, Fan CY, Patterson C, Cyr DM: **Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator**. *Cell* 2006, **126**(3):571-582.
471. Tomati V, Sondo E, Armirotti A, Caci E, Pesce E, Marini M, Gianotti A, Jeon YJ, Cilli M, Pistorio A *et al*: **Genetic Inhibition Of The Ubiquitin Ligase Rnf5 Attenuates Phenotypes Associated To F508del Cystic Fibrosis Mutation**. *Sci Rep* 2015, **5**:12138.
472. Sondo E, Falchi F, Caci E, Ferrera L, Giacomini E, Pesce E, Tomati V, Mandrup Bertozzi S, Goldoni L, Armirotti A *et al*: **Pharmacological Inhibition of the Ubiquitin Ligase RNF5 Rescues F508del-CFTR in Cystic Fibrosis Airway Epithelia**. *Cell Chem Biol* 2018, **25**(7):891-905.e898.
473. Kuang E, Okumura CY, Sheffy-Levin S, Varsano T, Shu VC, Qi J, Niesman IR, Yang HJ, Lopez-Otin C, Yang WY *et al*: **Regulation of ATG4B stability by RNF5 limits basal levels of autophagy and influences susceptibility to bacterial infection**. *PLoS Genet* 2012, **8**(10):e1003007.
474. Barrachina M, Maes T, Buesa C, Ferrer I: **Lysosome-associated membrane protein 1 (LAMP-1) in Alzheimer's disease**. *Neuropathol Appl Neurobiol* 2006, **32**(5):505-516.
475. Wang Q, Yao J, Jin Q, Wang X, Zhu H, Huang F, Wang W, Qiang J, Ni Q: **LAMP1 expression is associated with poor prognosis in breast cancer**. *Oncol Lett* 2017, **14**(4):4729-4735.
476. Lu M, Zhu H, Wang X, Zhang D, Xiong L, Zhu J, Mao Y, Qiang J: **LAMP1 expression is associated with malignant behaviours and predicts unfavourable prognosis in laryngeal squamous cell carcinoma**. *Pathology* 2016, **48**(7):684-690.
477. Sarafian VS, Koev I, Mehterov N, Kazakova M, Dangalov K: **LAMP-1 gene is overexpressed in high grade glioma**. *Apmis* 2018, **126**(8):657-662.

478. Lai KSP, Liu CS, Rau A, Lanctot KL, Kohler CA, Pakosh M, Carvalho AF, Herrmann N: **Peripheral inflammatory markers in Alzheimer's disease: a systematic review and meta-analysis of 175 studies**. *J Neurol Neurosurg Psychiatry* 2017, **88**(10):876-882.
479. Kim R, Kim HJ, Kim A, Jang M, Kim Y, Yoo D, Im JH, Choi JH, Jeon B: **Peripheral blood inflammatory markers in early Parkinson's disease**. *J Clin Neurosci* 2018, **58**:30-33.
480. Hall S, Janelidze S, Surova Y, Widner H, Zetterberg H, Hansson O: **Cerebrospinal fluid concentrations of inflammatory markers in Parkinson's disease and atypical parkinsonian disorders**. *Sci Rep* 2018, **8**(1):13276.
481. Dong Y, Li X, Cheng J, Hou L: **Drug Development for Alzheimer's Disease: Microglia Induced Neuroinflammation as a Target?** *Int J Mol Sci* 2019, **20**(3).
482. Gelders G, Baekelandt V, Van der Perren A: **Linking Neuroinflammation and Neurodegeneration in Parkinson's Disease**. *J Immunol Res* 2018, **2018**.
483. Roesch EA, Nichols DP, Chmiel JF: **Inflammation in cystic fibrosis: An update**. *Pediatr Pulmonol* 2018, **53**(S3):S30-s50.
484. Mulberg AE, Resta LP, Wiedner EB, Altschuler SM, Jefferson DM, Broussard DL: **Expression and localization of the cystic fibrosis transmembrane conductance regulator mRNA and its protein in rat brain**. *J Clin Invest* 1995, **96**(1):646-652.
485. Mulberg AE, Wiedner EB, Bao X, Marshall J, Jefferson DM, Altschuler SM: **Cystic fibrosis transmembrane conductance regulator protein expression in brain**. *Neuroreport* 1994, **5**(13):1684-1688.
486. Mulberg AE, Weyler RT, Altschuler SM, Hyde TM: **Cystic fibrosis transmembrane conductance regulator expression in human hypothalamus**. *Neuroreport* 1998, **9**(1):141-144.
487. Guo Y, Su M, McNutt MA, Gu J: **Expression and Distribution of Cystic Fibrosis Transmembrane Conductance Regulator in Neurons of the Human Brain**. *J Histochem Cytochem* 2009, **57**(12):1113-1120.
488. Marcorelles P, Friocourt G, Uguen A, Lede F, Ferec C, Laquerriere A: **Cystic fibrosis transmembrane conductance regulator protein (CFTR) expression in the developing human brain: comparative immunohistochemical study between patients with normal and mutated CFTR**. *J Histochem Cytochem* 2014, **62**(11):791-801.
489. Hector A, Schafer H, Poschel S, Fischer A, Fritzsching B, Ralhan A, Carevic M, Oz H, Zundel S, Hogardt M *et al*: **Regulatory T-cell impairment in cystic**

fibrosis patients with chronic pseudomonas infection. *Am J Respir Crit Care Med* 2015, **191**(8):914-923.
490. Siegmann N, Worbs D, Effinger F, Bormann T, Gebhardt M, Ulrich M, Wermeling F, Muller-Hermelink E, Biedermann T, Tighe M *et al*: **Invariant natural killer T (iNKT) cells prevent autoimmunity, but induce pulmonary inflammation in cystic fibrosis**. *Cell Physiol Biochem* 2014, **34**(1):56-70.
491. Hashiguchi K, Zhang-Akiyama QM: **Establishment of human cell lines lacking mitochondrial DNA**. *Methods Mol Biol* 2009, **554**:383-391.
492. Finucane OM, Sugrue J, Rubio-Araiz A, Guillot-Sestier MV, Lynch MA: **The NLRP3 inflammasome modulates glycolysis by increasing PFKFB3 in an IL-1β-dependent manner in macrophages**. *Sci Rep* 2019, **9**.
493. de Souza ABF, Chírico MTT, Cartelle CT, de Paula Costa G, Talvani A, Cangussú SD, de Menezes RCA, Bezerra FS: **High-Fat Diet Increases HMGB1 Expression and Promotes Lung Inflammation in Mice Subjected to Mechanical Ventilation**. *Oxid Med Cell Longev* 2018, **2018**.
494. Park YH, Oh EY, Han H, Yang M, Park HJ, Park KH, Lee JH, Park JW: **Insulin resistance mediates high-fat diet-induced pulmonary fibrosis and airway hyperresponsiveness through the TGF-β1 pathway**. *Exp Mol Med* 2019, **51**(5).
495. Saiyasit N, Chunchai T, Apaijai N, Pratchayasakul W, Sripetchwandee J, Chattipakorn N, Chattipakorn SC: **Chronic high-fat diet consumption induces an alteration in plasma/brain neurotensin signaling, metabolic disturbance, systemic inflammation/oxidative stress, brain apoptosis, and dendritic spine loss**. *Neuropeptides* 2020:102047.
496. Han X, Ding C, Zhang G, Pan R, Liu Y, Huang N, Hou N, Han F, Xu W, Sun X: **Liraglutide ameliorates obesity-related nonalcoholic fatty liver disease by regulating Sestrin2-mediated Nrf2/HO-1 pathway**. *Biochem Biophys Res Commun* 2020, **525**(4):895-901.
497. Bae SH, Sung SH, Oh SY, Lim JM, Lee SK, Park YN, Lee HE, Kang D, Rhee SG: **Sestrins Activate Nrf2 by Promoting p62-dependent Autophagic Degradation of Keap1 and Prevent Oxidative Liver Damage**. *Cell metabolism* 2013, **17**(1).
498. Gibson-Corley KN, Meyerholz DK, Engelhardt JF: **Pancreatic Pathophysiology in Cystic Fibrosis**. *J Pathol* 2016, **238**(2):311-320.
499. Twig G, Elorza A, Molina AJA, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh S, Katz S, Las G *et al*: **Fission and Selective Fusion Govern Mitochondrial Segregation and Elimination by Autophagy**. *The EMBO journal* 2008, **27**(2).

500. Li H, Liu S, Yuan H, Niu Y, Fu L: **Sestrin 2 induces autophagy and attenuates insulin resistance by regulating AMPK signaling in C2C12 myotubes**. *Exp Cell Res* 2017, **354**(1):18-24.
501. Larson-Casey JL, Deshane JS, Ryan AJ, Thannickal VJ, Carter AB: **Macrophage Akt1 kinase-mediated mitophagy modulates apoptosis resistance and pulmonary fibrosis**. *Immunity* 2016, **44**(3):582-596.
502. Bueno M, Lai YC, Romero Y, Brands J, St Croix CM, Kamga C, Corey C, Herazo-Maya JD, Sembrat J, Lee JS *et al*: **PINK1 deficiency impairs mitochondrial homeostasis and promotes lung fibrosis**. *J Clin Invest* 2015, **125**(2):521-538.
503. Burman JL, Pickle S, Wang C, Sekine S, Vargas JNS, Zhang Z, Youle AM, Nezich CL, Wu X, Hammer JA *et al*: **Mitochondrial Fission Facilitates the Selective Mitophagy of Protein Aggregates**. *The Journal of cell biology* 2017, **216**(10).
504. Aschner Y, Downey GP: **Transforming Growth Factor-beta: Master Regulator of the Respiratory System in Health and Disease**. *Am J Respir Cell Mol Biol* 2016, **54**(5):647-655.
505. Arkwright PD, Laurie S, Super M, Pravica V, Schwarz MJ, Webb AK, Hutchinson IV: **TGF-beta(1) genotype and accelerated decline in lung function of patients with cystic fibrosis**. *Thorax* 2000, **55**(6):459-462.
506. Drumm ML, Konstan MW, Schluchter MD, Handler A, Pace R, Zou F, Zariwala M, Fargo D, Xu A, Dunn JM *et al*: **Genetic modifiers of lung disease in cystic fibrosis**. *N Engl J Med* 2005, **353**(14):1443-1453.
507. Patel AS, Song JW, Chu SG, Mizumura K, Osorio JC, Shi Y, El-Chemaly S, Lee CG, Rosas IO, Elias JA *et al*: **Epithelial cell mitochondrial dysfunction and PINK1 are induced by transforming growth factor-beta1 in pulmonary fibrosis**. *PLoS One* 2015, **10**(3):e0121246.
508. Zhou Z, Torres M, Sha H, Halbrook CJ, Van den Bergh F, Reinert RB, Yamada T, Wang S, Luo Y, Hunter AH *et al*: **Endoplasmic reticulum-associated degradation regulates mitochondrial dynamics in brown adipocytes**. *Science* 2020, **368**(6486):54-60.
509. Li R, Ren T, Zeng J: **Mitochondrial Coenzyme Q Protects Sepsis-Induced Acute Lung Injury by Activating PI3K/Akt/GSK-3beta/mTOR Pathway in Rats**. *Biomed Res Int* 2019, **2019**:5240898.
510. Park JS, Davis RL, Sue CM: **Mitochondrial Dysfunction in Parkinson's Disease: New Mechanistic Insights and Therapeutic Perspectives**. *Curr Neurol Neurosci Rep* 2018, **18**(5).
511. Torsney KM, Forsyth D: **Respiratory dysfunction in Parkinson's disease**. *J R Coll Physicians Edinb* 2017, **47**(1):35-39.

512. Konstan MW, VanDevanter DR, Sawicki GS, Pasta DJ, Foreman AJ, Neiman EA, Morgan WJ: Association of High-Dose Ibuprofen Use, Lung Function Decline, and Long-Term Survival in Children with Cystic Fibrosis. *Ann Am Thorac Soc* 2018, 15(4):485-493.
513. Konstan MW, Wagener JS, Pasta DJ, Millar SJ, Jacobs JR, Yegin A, Morgan WJ: Clinical use of dornase alpha is associated with a slower rate of FEV1 decline in cystic fibrosis. *Pediatr Pulmonol* 2011, 46(6):545-553.
514. Bodas M, Vij N: Augmenting autophagy for prognosis based intervention of COPD-pathophysiology. *Respir Res* 2017, 18(1):83.
515. Pierzynowska K, Gaffke L, Cyske Z, Puchalski M, Rintz E, Bartkowski M, Osiadły M, Pierzynowski M, Mantej J, Piotrowska E *et al*: Autophagy stimulation as a promising approach in treatment of neurodegenerative diseases. *Metab Brain Dis* 2018, 33(4):989-1008.
516. Nemchenko A, Chiong M, Turer A, Lavandero S, Hill JA: Autophagy as a Therapeutic Target in Cardiovascular Disease. *J Mol Cell Cardiol* 2011, 51(4):584-593.
517. Cosin-Roger J, Canet F, Macias-Ceja DC, Gisbert-Ferrándiz L, Ortiz-Masiá D, Esplugues JV, Alós R, Navarro F, Barrachina MD, Calatayud S: Autophagy Stimulation as a Potential Strategy Against Intestinal Fibrosis. *Cells* 2019, 8(9).
518. Moreira PI, Zhu X, Wang X, Lee H, Nunomura A, Petersen RB, Perry G, Smith MA: Mitochondria: A Therapeutic Target in Neurodegeneration. *Biochim Biophys Acta* 2010, 1802(1):212-220.
519. Pustylnikov S, Costabile F, Beghi S, Facciabene A: Targeting mitochondria in cancer: current concepts and immunotherapy approaches. *Transl Res* 2018, 202:35-51.
520. Quan Y, Xin Y, Tian G, Zhou J, Liu X: Mitochondrial ROS-Modulated mtDNA: A Potential Target for Cardiac Aging. *Oxid Med Cell Longev* 2020, 2020:9423593.