SAPIENZA
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Microbicidal mechanisms of human macrophages against *Pseudomonas aeruginosa* in healthy subjects and cystic fibrosis patients

Dottorato di ricerca in Biologia Cellulare e dello Sviluppo
XXV ciclo

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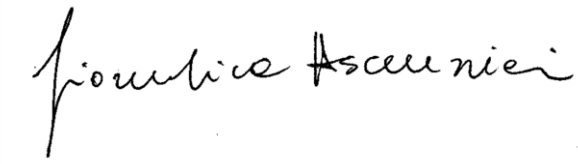

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Cystic fibrosis

Cystic fibrosis (CF) is one of the most common genetic diseases affecting Caucasian population showing an incidence of 4.0/10.000 (i.e. 1/2500) live births and particularly 2.9/10.000 (i.e. 1/3500) in Europe (Scotet V et al, 2012). CF was originally described in 1938 as mucoviscidosis in babies with pancreatic insufficiency that failed to grow and often succumbed to pulmonary infection in infancy or early childhood. Only in 1989 the gene responsible of CF was identified and named cystic fibrosis trans-membrane conductance regulator (CFTR) by Riordan and colleagues (Riordan JR, 1989) (Davis PB, 2006).

Since the discovery of CFTR, enormous progresses have been made in the treatment and management of CF patients leading to substantial increase of life expectancy (figure 1). This was mainly due to combinations of early diagnosis, antibiotic treatments, anti-inflammatory therapy and pancreatic enzyme supplements (Davis PB, 2006). Nevertheless, the progression of pulmonary symptoms with destruction of pulmonary tissue cannot be controlled sufficiently and lung disease still represents the major cause of mortality and morbidity in CF patients (Gaggar A et al, 2011).

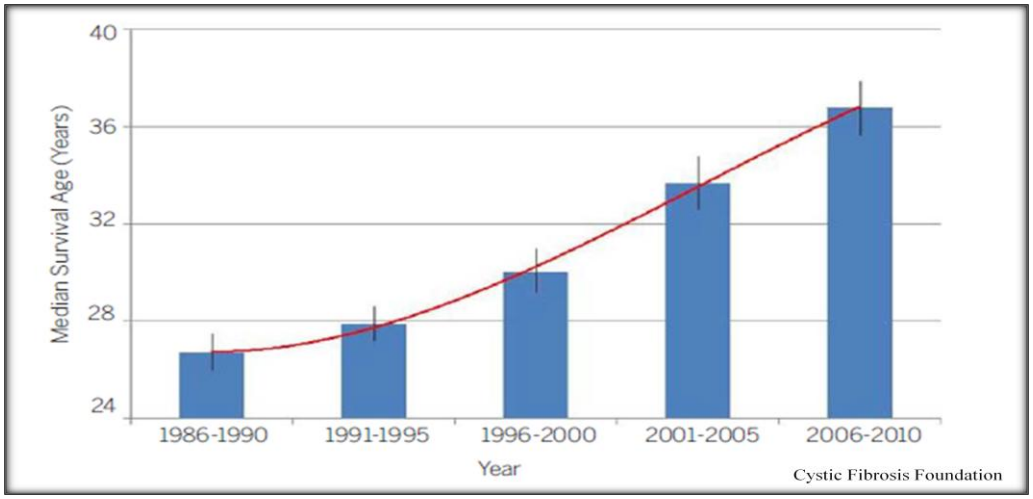


***Figure 1**. Median predicted survival age, 1986-2010.*

The CFTR gene

The CFTR gene was discovered in 1989 using the positional cloning approach by three research groups leaded by Lap-Chee Tsui and Jack Riordan at the Hospital for Sick Children in Toronto and Francis Collins at the University of Michigan. The CFTR gene is located on the long arm of chromosome 7 at position 7q31, it spans approximately 190 kb of genomic

DNA and consists of 27 exons. It encodes a 6,5Kb transcript that directs the synthesis of a 1480 amino acids trans membrane protein that functions as chloride channel (Ellsworth RE et al, 2000; McCarthy VA and Harris A, 2005; Davis PB, 2006; Rogan MP et al, 2011).

Approximately 1.600 different disease associated mutations have been identified so far. They are categorized into six classes based on their effects on the CFTR transcription, intracellular protein processing and trafficking and channel activity (figure 2) (Rogan MP et al, 2011).

CFTR mutations of classes I, II and III cause substantial decline in CFTR expression or function and are associated with pancreatic insufficiency, whereas classes IV, V and VI mutations have some residual CFTR activity and are often associated with pancreatic sufficiency. CFTR mutations can be classified as mild or severe depending on whether they result in pancreatic insufficiency or not (Zielenski J, 2000). However, other factors such as the environment, therapeutics and other genes (modifier) can contribute to the outcome of the CF disease (Davis PB, 2006). Indeed, the phenotypic expression of CFTR mutations can vary greatly and the correlation between pulmonary disease and the genotype has not been completely understood (Corey M, et al, 1997).

Among CF mutations ΔF508, a single codon deletion at position 508, is by far the most widespread mutations being present on at least one allele in approximately 70-90% of the patients (Riordan JR, 2008). To date, the majority of CF mutations are rare and have not been functionally characterized, in effect besides ΔF508 only four specific mutations reach a frequency of 1% to 3%: G551D, W1282X, G542X and N1303K (Castellani C et al, 2008).

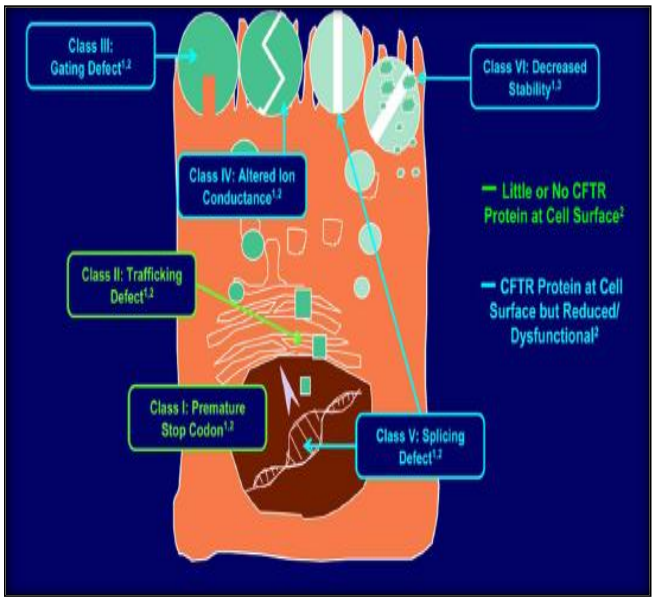


Figure 2. *CFTR mutation classes.*

The CFTR protein and its activity/ies

The wild-type CFTR glycoprotein localizes to the apical membrane of epithelial cells where it functions as a cAMP-regulated chloride channel. It is essential to maintain ion and fluid homeostasis in secretory and resorptive epithelium (Riordan JR, 1989; Quinton PM, 2008). The absence, or the impaired activity of CFTR results in the failure of chloride ions to exit the cells leading to an imbalance of counterions and water movement which causes dehydration of secretions. This occurs in almost all exocrine tissues but the most serious consequences on organ function are detected in the pancreas and in the lung. CFTR dysfunctions in the sweat duct block salt reabsorption (Riordan JR, 2008). Although, mainly expressed by epithelial cells, CFTR expression was also reported in cardiac myocytes (Nagel G et al,1992), smooth muscle (Robert et al, *2*005), endothelia (Tousson A et al, 1998), erythrocytes (Sprague RS et al, 1998) and immune cells (Khan TZ et al, 2005).

CFTR belongs to the ATP-binding cassette (ABC) transporter protein family but it is the only member of this large protein family known to function as an ion channel. CFTR consists of two homologous nucleotide binding domains (NBDs) and two membrane spanning domains (MSDs), each composed of six trans-membrane domains connected by intra and extracellular loops. The presence of a charged intra-cytoplasmic regulatory domain (R domain) distinguishes CFTR from ABC transporters. The MSDs assemble to form a low conductance anion-selective pore and the NBDs form a head-to-tail dimer with two ATP-binding sites located at the dimer interface. The R domain is phosphorylated by cAMP-dependent protein kinase A (PKA) (Hwang TC and Sheppard DN, 2009).

Chloride transport by CFTR requires the interaction among its multiple domains and phosphorylation of the R domain by PKA. Phosphorylated R domain alters its conformation as well as the contacts with other protein domain (Dulhanty AM and Riordan JR,1994; Ostedgaard LS et al, 2001; Baker JM et al, 2007; Grimard V et al, 2004) and controls the channel opening (Davis PB, 2006). Conformational movements within and between the two NBDs are coupled to rearrangements among MSDs shifting the equilibrium between the open or closed ion pore. The binding and the hydrolysis of the ATP ligand and channel gating has not yet been entirely elucidated although it has been reported that the interaction of ATP with one of the two NBDs powering conformational changes in the MDSs opening channel pore. ATP hydrolysis promotes dissociation of the NBDs, resulting in a return to the closed

configuration (Lewis HA et al, 2004; Vergani P eet al, 2005; Aleksandrov AA et al, 2007; Riordan JR, 2008).

According to this model, ATP is not required to transport Cl^- across plasma membrane but only to allows conformational changes in the protein that in turn open the gate and the phosphorylated R domain regulates anion flow through the CFTR pore (Hwang TC and Sheppard DN, 2009).

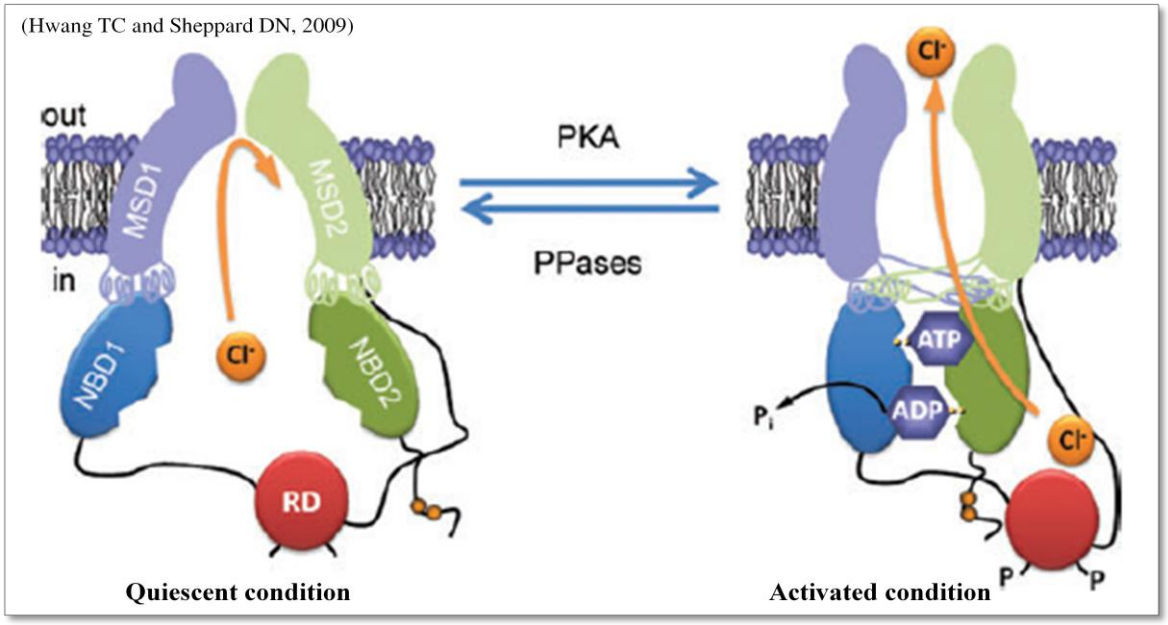


Figure 3. *Simplified CFTR gating model.*

In addition to chloride-channel activity, it has been proposed that the CFTR has other roles that directly or indirectly impact other proteins and cell functions.

The CFTR plays an important role in $HCO3^-$ secretion because it is permeable to the anion and stimulates $Cl^-/HCO3^-$ exchangers. Loss of this activity causes impaired pancreatic $HCO3^-$ secretion in CF patients, but it also causes pH reduction of the epithelial surface liquid (Devor DC et al, 1999; Choi JY et al, 2001). Additionally, prevention of the passage of other permeant species in CF has been reported such as the failure to transport the thiocyanate anion to the surface of CF airway epithelium, which in turn causes reduction of antibacterial hypothiocyanite, which normally contribute to eliminate *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Moskwa P et al, 2007).

CFTR has been shown to transport glutathione (GSH) in artificial systems, whether or not this occurs physiologically is still controversial. CFTR may not actually conduct GSH, but may

regulate its transport indirectly through chloride transport. Nevertheless, decreased GSH levels in lung epithelial lining fluid and blood plasma of CF patients have been reported, thus contributing to increased inflammation and infections present in the CF lungs (Ballatori N et al, 2009).

CFTR also regulates other channels, transporters, receptors and signaling molecules. Experimental evidences suggest that CFTR negatively regulates the conductive epithelial Na^+ permeability mediated by the epithelial sodium channel (ENaC). Thus, the lack of CFTR activity causes a continued or enhanced Na^+ absorption that is the major cause of airway surface liquid dehydration and impaired mucociliary clearance. Despite the down-regulation of ENaC by CFTR has been experimentally confirmed, the detailed mechanisms of their interactions are not yet understood (Toczyłowska-Mamińska R and Dolowy K, 2012).

Additionally, CFTR can activate the Na^+/H^+ and HCO_3^-/Cl^- exchangers, the KvLQT1 channel and the sodium potassium pump. Finally, the basolateral outwardly rectifying chloride channel (ORCC) and CFTR have been reported to activate the aquaporin AQP3 thus coupling $Cl^-$and water transport (Toczyłowska-Mamińska R and Dolowy K, 2012).

Lung disease

Chronic lung disease, characterized by recurrent cycles of infection and inflammation throughout the patient's life, is the major cause of death in patients with CF (Davis PB, 2001). Longitudinal and prospective studies of infant with CF demonstrated that CF airways are not infect at birth, in contrast to the pancreas and the gut that are often damaged (Davis PB, 2006). However, opportunistic bacteria enter the lung from the environment and then, possibly favored by impaired activity of the innate immune cells, established chronic infection. Despite intensive antimicrobial therapies, CF airway infections are only transiently suppressed and never eradicated with consequent permanent lung colonization. The infections are usually confined to the airway lumen and sepsis is an extraordinarily rare event indicating that impairment of the local defenses mainly contribute to lung disease (Sorio C and Melotti P, 2009; Worlitzsch D et al, 2002). Although it is clear that the primary cause of CF disease is the lack of normal levels of functional CFTR protein, the mechanisms by which this leads to chronic lung infection have not been fully elucidated. Different hypothesis have been formulated to link the defective airway defence in CF to defective ion transport. First, the "high salt hypothesis", suggests that airway epithelia whit functional CFTR produce low NaCl airways surface liquid (ASL) (relative to plasma) that permits efficient activity of secreted

antimicrobial substances. In CF, the absence or dysfunctional CFTR causes the production of a "high NaCl" ASL (equal to plasma) that inhibits the activity of antimicrobial peptides on airway surfaces leading to chronic infection (Smith JJ et al, 1996; Zabner J et al, 1998). In marked contrast to this hypothesis, a second one the "too little salt" or "low ASL volume", suggests that the CFTR protein works to coordinately control the relative rates of airway epithelial Na^+ absorption and Cl^- secretion and that the absence of these CFTR-regulated activities leads to an increase of Na^+ and Cl^- within the cells and water driven by osmotic force. Consequently ASL becomes dehydrated and favors adhesion of mucus to airway surfaces, airways obstruction and chronic infection (Knowles MR and Boucher RC, 2002; Tarran R et al, 2001) (figure 4). Evidence from *in vitro* and *in vivo* models, and recent human clinical studies favor the "too little salt or dehydration hypothesis" that at present represents the most useful guide to study CF pathogenesis and therapeutic approaches (Boucher RC, 2007).

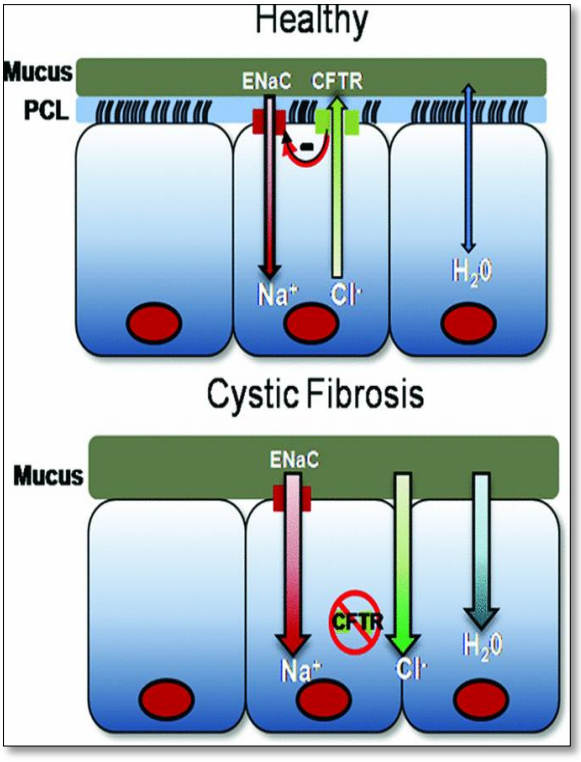


Figure 4. *CFTR impact on ASL composition . The ASL is composed by mucus and periciliary liquid (PCL). Normally, the ASL is 2% solids (1% salt; 1% mucin; 98% water), and it functions as a bacteria and particle trap which are eliminated by the propulsive activity of cilia that move the PCL . In contrast, in CF, the depletion of water leads to a reduction and dehydration of PCL with consequently adhesion of the mucus layer to the airway surface and impaired mucociliary clearence.*

In addition to the altered composition of ASL other factors contribute to the establishment of chronic infection and inflammation. For example, the ratio of asialylated to sialylated glycolipids is higher in CF cells compared to non-CF cells providing additional receptors for *P. aeruginosa* and *Burkholderia cepacia* complex (Bcc), two of the most important pathogens in CF (Callaghan M and McClean S, 2012). It has been proposed that CFTR acts as a receptor for *P. aeruginosa* thus leading to bacterial internalization and subsequent clearance of this pathogen. In this model, the binding of the microbe to the CFTR is required to promote its internalization, to stimulate cytokine secretion and ultimately to kill the bacteria (Khan TZ et al, 1995; Di A et al, 2006; Kowalski MP et al, 2007).

Beside the alteration of the ASL, it has been postulated that the lack of CFTR activity impacts the immune response in the respiratory tract at multiple levels. Inflammation in the murine CF lung has also been correlated to ceramide accumulation (Dubin PJ et al, 2007) accordingly, in CF patients it has been observed accumulation of ceramide in nasal epithelial cells, respiratory epithelial cells and submucosal glands that could contribute to the establishment of chronic infections (Teichgraber V et al, 2008). Many humoral components of immunity are inefficent in CF, possibly due to dehydrated ASL (Boucher RC, 2007) and to reduced Cl^- secretion onto the epithelia by the submucosal glands (Verkman AS et al, 2003; Joo NS et al, 2004). Additionally, impaired activity of bone marrow derived immune cells have been implicated in CF lung disease. Defective phagocytosis has been reported to occur in neutrophils and macrophages collected from bronchoalveolar lavage fluid in CF patients (Alexis NE et al, 2006). It also been reported that defective CFTR affects neutrophils functions leading to a decreased ability to kill microrganisms (Painter RG et al, 2006; Painter RG et al, 2008) and increased resistance to apoptosis that is required to end up the inflammatory response (Dimagno MJ et al, 2005). Macrophages, which are the main subjects of my PhD work, have also been related to CF lung disease and will be discussed in the next section. Finally in CF, abnormal secretion of proteases compromises lung functions and the immune response to pathogens. In particular, neutrophil elastase predominates airways where it destroys a vast range of anti-microbial compound, degrades mucin and induces IL-8 secretion. Dysregulation of the matrix metalloproteases (MMPs) in CF has also been proposed to contribute to lung failure and to bacteria colonization (Henke MO et al, 2011; Cosgrove S et al, 2011).

In summary, the CFTR mutations lead to defective regulation of chloride and sodium transepithelial ion flux, resulting in increased water absorption, dehydration of ASL, impaired mucociliary clearance and altered response of the innate immune cells. Consequently, all these mechanisms contribute to the chronic infections and inflammation that lead to progressive lung disease and ultimately pulmonary failure, which is the primary cause of death in CF patients (Boucher RC, 2007; Lipuma JJ, 2010).

Macrophages

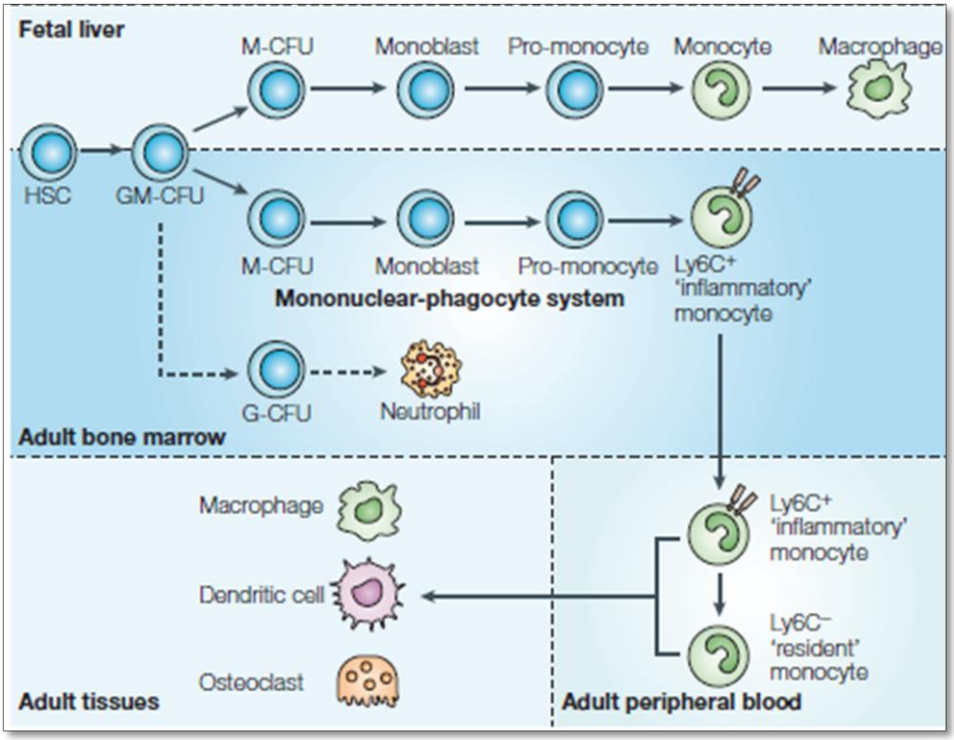
Macrophages are terminally differentiated phagocytic cells belonging to the mononuclear phagocyte system that also comprises dendritic cells (DCs), circulating blood monocytes and committed myeloid progenitor cells in the bone marrow (Cassetta L et al, 2011). Macrophages differentiate from circulating peripheral blood mononuclear cells (PBMCs), which migrate into tissues in the steady state or in response to inflammation. PBMCs develop from a common myeloid progenitor cell in the bone marrow that is the precursor of many different cell types, including neutrophils, eosinophils, basophils, macrophages, DCs and mast cells (figure 5).

During monocyte development, myeloid progenitor cells (termed granulocyte/macrophage colony-forming units) sequentially give rise to monoblasts, pro-monocytes and finally monocytes, which are released from the bone marrow into the bloodstream. Monocytes migrate from the blood into tissues to replenish long-lived tissue-specific macrophages (Gordon S and Taylor PR, 2005).

In addition, it has been reported that tissue-resident macrophage populations can contribute to the renewal and maintenance of different macrophage types, particularly under steady-state conditions. Inflammatory insults, such as trauma or infection, usually increase the recruitment of monocytes to aid macrophages repopulation in the tissues (Gordon S and Taylor PR, 2005).

Macrophages have many roles in homeostasis maintenance, including host defence, tissue remodelling and repairing (Cassetta L et al, 2011; Mosser DM and Edwards JP, 2008). Many of these activities appear to be opposite: pro versus anti-inflammatory effects, immunogenic versus tolerogenic activities and tissue destruction versus tissue-repair suggesting that the functional pattern expressed by macrophages changes with time as the response progresses (Martinez FO et al, 2006; Martinez FO et al, 2009; Hume DA, 2008).

The plethora of functions performed by macrophages reflects their plasticity that allows them to efficiently respond to environmental signals and to change their phenotype and their physiology (Mosser DM and Edwards JP, 2008). Macrophages can respond to high number of stimuli including cytokines, chemokines, hormones (including adrenergic and cholinergic agonists), TLR ligands and other endogenous ligands (e.g. histamine, integrin ligands, peroxisome proliferator-activated receptor ligands, apoptotic cells). Furthermore, identical macrophages placed in different microenvironments display different functions in response to a common stimulus (Cassetta L et al, 2011). Macrophages can be markedly altered by both innate and adaptive immune response, moreover macrophages themselves can produce several factors that influence their own physiology (Mosser DM and Edwards JP, 2008).



(Gordon S and Taylor PR, Nat Rev Immunol, 2005)

Figure 5. *Mononuclear phagocyte system.*

Monocytes originate in the bone marrow from a common haematopoietic stem cell (HSC). They undergo differentiation steps during which they commit to the myeloid and then to a monocyte lineage. In response to macrophage colony-stimulating factor, they divide and differentiate into monoblasts and then pro-monocytes before becoming monocytes, which exit the bone marrow and enter the bloodstream.

Macrophages have been functionally classified into M1 (classical activated), M2 (alternatively activated) and regulatory, in an effort to emulate the T-cell literature (Figure 6). T cells principally regulate macrophages polarization by cytokines production. Nevertheless, most Th1 and Th2 cytokines do not seem to induce a stable differentiation of macrophages into distinct subsets, but they rather promote a transient functional pattern of responses that return to basal levels in few (3–7) days (Cassetta L et al, 2011).

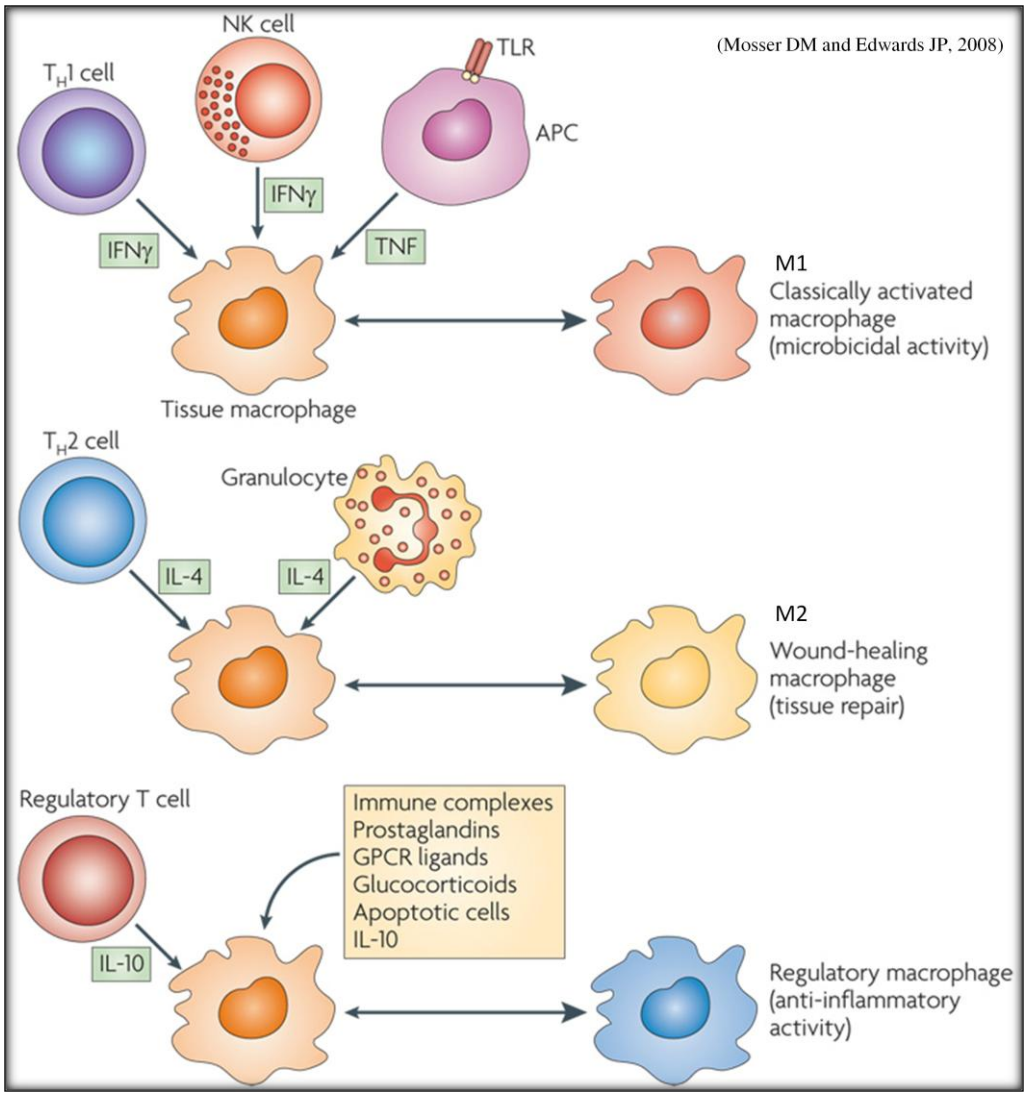


Figure 6. *Macrophage polarization.*

Classically activated macrophages arise in response to interferon-γ (IFNγ), which can be produced during an adaptive immune response by T helper 1 (TH1) cells or CD8+ T cells (not shown) or during an innate immune response by natural killer (NK) cells, and tumour-necrosis factor (TNF), which is produced by antigen-presenting cells (APCs). Wound-healing (alternatively activated) macrophages arise in response to interleukin-4 (IL-4), which can be produced during an adaptive immune response by TH2 cells or during an innate immune response by granulocytes. Regulatory macrophages are generated in response to various stimuli, including immune complexes, prostaglandins, G-protein coupled receptor (GPCR).

Macrophage plasticity represents an adaptive response to the different environments in which macrophages migrate in response to different stimuli. Both innate and adaptive signals can influence macrophage physiology and these alterations allow them to participate to homeostastic processes, such as tissue remodeling, wound healing and host defence. However, each of these activities can have potentially dangerous consequences if not appropriately regulated.

Phagocytosis

Macrophages are professional phagocytes and as such are the uniquely qualified cells to perform phagocytosis. Phagocytosis is a critical component of the innate and adaptive immune responses to pathogens, in addition it has been reported to be crucial for tissue homeostasis and remodeling. Phagocytosis is defined as the ingestion by cells of large particles ($\geq$ 0.5 μm). Phagocytes are able to engulf both foreign bodies and endogenous (apoptotic) corpses. These processes lead to very different outcomes and they are dissimilar for some aspects such as for example the receptors involved in particle recognition.

Foreign bodies, such as bacteria or fungi, can be cleared from infection sites by professional phagocytes. Moreover, these cells can present to lymphoid cells antigens derived from the degradation of engulfed particles. Thereby, phagocytosis contributes to the first line of defense against infections and it also plays a key role in the initiation of the adaptive immune response. (Flannagan RS et al, 2009; Flannagan RS et al, 2012).

Effective phagocytosis requires three components: particle recognition, particle internalization and phagosome maturation.

Particle recognition

The interaction of the microorganisms with the phagocytes can be direct, through recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), or indirect, through mediation by opsonins (Flannagan RS et al, 2009).

PAMPs can be detected by several receptors, in particular by Toll like receptors (TLRs), but also by some phagocytic receptors (Table 1). In some cases these receptors, such as Dectin-1, suffice to trigger phagocytosis. In other cases the contribution of the receptors to phagocytosis is indirect, it occurs either by tethering the prey to the cell surface or by priming the phagocytic response that is mediated by other receptors (Doyle SE et al, 2004).

Opsonins are host factors, such as immunoglobulin G (IgG) and components of the complement cascade, that attach to the pathogen surface acquiring a conformation recognized by opsonic receptors, such as Fcγ receptors (FcγRs) and complement receptor 3 (CR3) (Roitt I, 1994).

The receptor-ligand interaction is dictated by their mutual affinity and by their density on the surface of the phagocyte and prey. Usually, multiple opsonic and nonopsonic receptors are engaged simultaneously, producing a complex and probably synergistic response. Engagement of multiple receptors is facilitated by the dynamic nature of phagocytes, which continuously extend actin-dependent membranous projections to probe their surroundings (Kusner DJ et al, 1999; Holevinsky KO and Nelson DJ, 1998; Bajno Let al, 2000).

Table 1. *Human receptor mediating phagocytosis and their ligands.*

Receptors	Ligands
Pattern-recognition receptors	
Mannose receptor (CD206)	Mannan
Dectin-1 (CLEC7A)	β1,3-glucan
CD14	Lipopolysaccharide-binding protein
Scavenger receptor A (CD204)	Lipopolysaccharide, lipoteichoic acid
CD36	*Plasmodium falciparum*–infected erythrocytes
MARCO	Bacteria
Opsonic receptors	
FcγRI (CD64)	IgG1 = IgG3 > IgG4
FcγRIIa (CD32a)	IgG3 ≥ IgG1 = IgG2
FcγRIIc (CD32c)	IgG
FcγRIIIa (CD16a)	IgG
FcαRI (CD89)	IgA1, IgA2
FcεRI	IgE
CR1 (CD45)	Mannan-binding lectin, C1q, C4b, C3b
CR3 ($\alpha_M\beta_2$, CD11b/CD18, Mac-1)	iC3b
CR4 ($\alpha_V\beta_2$, CD11c/CD18, gp150/95)	iC3b
$\alpha_5\beta_1$	Fibronectin, vitronectin
Apoptotic corpse receptors	
TIM-1	Phosphatidylserine
TIM-4	Phosphatidylserine
BAI1	Phosphatidylserine
Stabilin-2	Phosphatidylserine
Mer	Gas6, protein S
$\alpha_V\beta_3$	MFG-E8
$\alpha_V\beta_5$	Apoptotic cells
CD36	Oxidized lipids

(Flannagan RS et al, 2011)

Particle internalization

The signaling cascade triggered by the ligand-receptor interaction varies depending on the nature of the receptor engaged. The pathway elicited by FcγR is well known. Following binding, clustering of the FcγR induces a signaling cascade leading to the engulfment of the particle by an actin-driven process. This process, that is replicated numerous times, leads to additional encounters between unoccupied receptors and available ligands on the surface of the particle, which contributes to complete internalization (Flannagan RS et al, 2012). In details, exposure to multivalent ligands induces clustering of FcγR and moving of their cytosolic domains into close proximity. The cytosolic domains encompass a unique immunoreceptor tyrosine based activation motif (ITAM), that is the substrate for phosphorylation by tyrosine kinases of the Src family (SFK). The

mechanism whereby receptor clustering leads to phosphorylation of the ITAM tyrosines remains elusive. Clustering may induce association between the receptors and cholesterol-enriched lipid rafts, where SFK kinases are concentrated. ITAM phosphorylation recruits and activates the spleen tyrosine kinases (SYK), which in turn phosphorylates various substrates (Daëron, M., 1997). SYK leads to the recruitment of additional signaling proteins to the activated FcγR complex. Several adaptors bind directly to the tyrosine kinases and act as platforms for the recruitment of downstream signaling components. The resulting signaling platforms activate multiple lipid-modification enzymes and guanine nucleotide exchange factors for small GTPases, two important components of actin remodeling during phagocytosis (Flannagan RS et al, 2012).

Lipids play a critical role in orchestrating the signaling events that trigger phagocytosis. Both phosphatidylinositol-4,5-bisphosphate (PI(2)P) and phosphatidylinositol- 3,4,5-trisphosphate (PI(3)P) accumulate at sites of particle engagement and are instrumental in timing the onset and termination of actin assembly. Whereas PI(2)P is essential for the initial polymerization that drives pseudopod formation, its conversion to PI(3)P seems to be required for pseudopod extension and phagosomal closure, at least in part by recruitment of myosin. Several enzymes contribute to the elimination of (PI(2)P); the phosphoinositide-specific phospholipase Cγ (PLCγ) is phosphorylated and recruited to the phagocytic cup in a SYK-dependent manner, probably by interaction of its SH2 domain with adaptor proteins. Phospholipases A and D are also necessary for successful completion of phagocytosis although their precise role has not been fully resolved (Flannagan RS et al, 2009; Flannagan RS et al, 2012).

Several GTPases play a role in FcγR mediated phagocytosis. Cdc42, which stimulates the formation of filopodia in various cell types, is activated early in phagocytosis, mostly at the rims of the cup. Rac1 and Rac2, which induce the formation of lamellipodia, are also stimulated although with different kinetics. Shortly after Cdc42 is stimulated, Rac1 is activated throughout the entire nascent phagosome whereas Rac2 is activated later, mostly at the base of the cup (Hoppe AD and Swanson JA, 2004). Rho-family GTPases are not the only small GTPases involved in phagosome formation. ARF6 is activated early in FcγR-mediated phagocytosis and promotes the delivery of endomembranes to the nascent phagosome (Niedergang F et al, 2003; Beemiller P et al, 2006).

The actin remodelling process requires the activity of the nucleator complex Arp2/3 that supports actin polymerization and promotes formation of branched actin filaments. Arp2/3 can be activated by the nucleation-promoting factors such as the Wiskott-Aldrich syndrome protein (WASP)/N-WASP and the Scar/WAVE family proteins. Although the actin polymerization is required for phagocytosis its sustained polymerization prevents internalization particularly of large particles indeed, it has to be cleared from the base of the cup prior to sealing. This process requires the

disappearance of PI(2)P, which is mediated by PLCγ and stimulated by PI3K (phosphatidylinositol 3-kinase). Removal of actin from the base of the phagosome may eliminate a possible barrier for membrane deformation, allowing the membrane bound particle to sink into the cytoplasm. Myosins also play a role in the particle engulfment process. In particular myosins II, IXb, and IC have been detected on forming phagosomes and myosin X has been demonstrated to participate to phagosome formations as effector of PI3K (May RC et al, 2000; Larsen EC et al, 2002).

During phagocytosis of large or multiple particles, a considerable amount of membrane is internalized, and the cell needs to compensate for the loss of surface area. Although the phagosomal membrane derives mostly from the plasma membrane, membranes from several intracellular compartments are also used (Touret N et al, 2005). In particular, recycling endosomes and late endosomes fuse with the phagocytic cup, as indicated by the delivery of the specific markers vesicle-associated membrane protein 3 (VAMP3) and/or Rab11 and TIVAMP/ VAMP7, but even lysosomes have been reported to fuse when the demand for membrane is excessive. Membrane delivery is important because inhibition of exocytosis reduces phagocytosis in a size dependent manner moreover it has been reported that the plasmalemmal surface in fact increases during phagocytosis (Holevinsky, K. O and Nelson DJ, 1998; Bajno L et al, 2000; Cox D et al, 2000; Braun V et al, 2004).

Dectin-1 has been identified as an important phagocytic receptor for fungi. Its cytosolic domain bears an ITAM-like motif, suggesting that it shares signaling features with FcγRs.

CR3, also known as αMβ2 integrin or Mac-1, is one of the best-studied phagocytic receptors. Early studies of CR3-mediated phagocytosis by electron microscopy suggested that particles sink into the phagocyte without extending the pseudopods that are characteristic of FcγR phagocytosis. However, this difference has been questioned by more recent observations from both electron and light microscopy studies, which showed membrane protrusions encircling the targets during CR3-mediated phagocytosis (Hall AB et al, 2006; Patel PC and Harrison RE, 2008; Bohdanowicz M et al, 2010). During CR3-mediated phagocytosis, F-actin accumulation and particle uptake depend on RhoA and are independent from Rac or Cdc42. RhoA activates actin polymerization by two distinct mechanisms: it activates Rho kinase which phosphorylates myosin II which in turn recruits Arp2/3 and activates the actin nucleator mDia1, recruited to the phagocytic cup. Thus microtubules, together with two types of actin nucleators Arp2/3 and mDia1, function cooperatively in CR3-mediated phagocytosis. The mechanism leading to RhoA activation is not fully understood. CR3 could activate RhoA in a tyrosine kinase-independent manner or, possibly, via SYK (May RC et al, 2000; Olazabal IM et al,2002; Colucci-Guyon E et al, 2005).

In summary particle internalization by phagocytes is a complex process requiring actin polymerization which is triggered by different pathways depending on the receptor engaged.

Phagosome maturation

Immediately after the scission from the surface membrane the phagosome is disarmed but it becomes a potent microbicidal organelle by a rapid succession of biochemical modification termed phagosome maturation (figure 7). This remodeling process starts immediately after and possibly even before phagosome sealing, it radically alters the composition of the phagosome while keeping its size nearly constant. Regardless the precise mechanisms, the sequential interactions with discrete endosomal compartments change the phagosome composition making its lumen markedly acidic (luminal pH ≈ 4.5), highly oxidative and enriched with hydrolytic enzymes that ultimately degrade its contents. By the maturation process, the innocuous phagosome is converted into the phagolysosome, a potent microbicidal organelle that is central to both innate and adaptive immunity (Flannagan RS et al, 2012).

The early phagosome

Newly formed phagosomes rapidly gain many of the properties of early endosomes. Their lumen is mildly acidic (pH 6.1–6.5) and poor in hydrolytic activity (Flannagan RS et al, 2009). During maturation the early phagosomes, generated through fusion of nascent phagosomes with early endosomes, acquire the Small GTPase-Rab5A that coordinates endocytic traffic and early phagosome biogenesis. The mechanism that triggers early recruitment of Rab5A to phagosomes is not fully elucidated. It has been reported that some active Rab5 exists on the plasmalemma prior

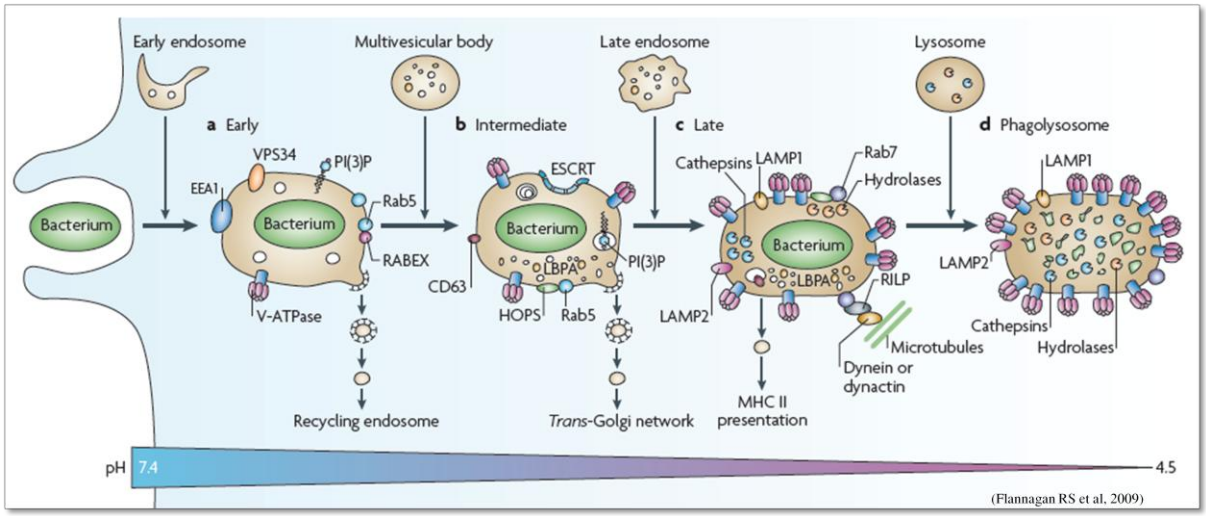


Figure 7. *Stages of phagosome maturation.*

to particle engulfment but the density of Rab5 on the phagosome membrane is much greater than detectable at the plasmalemma, so additional recruitment must occur. Rab5A stimulates the fusion of nascent phagosomes with early endosomes, also known as sorting endosomes (Vieira OV et al,

2003). Rab5A acts using multiple effectors, including the p150–hvPS34 complex, early endosome antigen 1 (EEA1) and SNARE proteins. The p150 kinase supports the recruitment of hvPS34, a class III phosphatidylinositol-3-kinase, which generates (PI(3)P) on the early phagosome membrane (Vieira OV et al, 2001). PI(3)P accumulation facilitates subsequent steps in phagosome maturation bearing effector proteins to the cytosolic face of the phagosome, such as the p40 subunit of the NADPH oxidase, the hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs) (Fratti RA et al, 2001; Vieira OV et al, 2004; Ellson C et al, 2006) and EEA1 (early end ant 1). EEA1 is thought to act as a bridge that tethers early endosomes to incoming endocytic vesicles (Callaghan J *et al*, 1999; Lawe DC et al, 2000) and probably has an equivalent role in phagosomes. Additionally, EEA1 also interacts directly with Rab5A, with syntaxin 13, a SNARE protein required for membrane fusion, and with an *N*-ethylmaleimide-sensitive fusion protein that is essential for the disassembly and reuse of SNARE complexes (McBride HM et al *1999;* Mills IG, 2001). As a consequence of these initial fusion events, early phagosomes become biochemically distinct.

Other cellular factors involved in the retrieval of components from endosomes and presumably from phagosomes are the Eps15 homology domain proteins (EHDs) and the hetero-oligomeric complex COPI. EHDs are lipid-binding proteins that stimulate membrane tubulation and influence recycling from Rab4 and Rab11-positive endosomes (Naslavsky N et al, 2006; Grant BD and Caplan S, 2008; Sharma M et al, 2008). The GTPase adenosine ribosylation factor COPI, and the retromer (a multisubunit protein sorting machine), are also required for efficient retrieval of endosomal and phagosomal proteins to either the plasmalemma or the *trans*-Golgi network (TGN) via recycling pathways (Botelho RJ et al, 2000; Beron W et al, 2001; Niedergang F et al 2003; Arighi CN et al, 2004).

Membrane budding and the emergence of recycling compartments from early phagosomes are not the sole mechanism that contribute to the maintenance of phagosome size. Phagosomes also divert membrane-associated cargo that is destined for degradation to intraluminal vesicles (ILVs). Such vesicles are thought to arise from inwards budding and pinching of the limiting membrane of the phagosome, in a manner akin to the generation of multivesicular bodies (MVBs). As in endosomes, phagosomal membrane proteins destined for degradation are ubiquitinated and associate with the endosomal-sorting complex required for transport (ESCRT) (Lee WL et al, 2005).

In summary an elaborate network of signaling, budding and tubulating components regulate the fusion and fission events underling the maturation of nascent phagosome into early phagosome.

The late Phagosome

Once the recycling proteins are removed, the phagosomes proceed to a more mature stage that is defined by the acquisition of distinct biochemical markers, including the small GTPase Rab7, with

the concomitant loss of early markers such as Rab5A. Compared with early phagosomes, late phagosomes are more acidic (pH 5.5–6.0), which is a consequence of the acquisition of additional proton pumps. Proton pumping is catalyzed by the vacuolar ATPase (V-ATPase), a multimeric protein complex that translocates H^+ (inward) across endosomal and phagosomal membranes at the expense of ATP. The late phagosomes are also enriched in proteases and lysosomal-associated membrane proteins (LAMPs), which are either imported from the Golgi complex or acquired by fusion with late endosomes (Flannagan RS et al, 2009; Flannagan RS et al, 2012).

The mechanisms whereby an early Rab5- positive/Rab7-negative phagosome becomes a late Rab7-positive/Rab5-negative phagosome are still little known but some critical molecular players that mediate this transition have been identified. In the *C. elegans* model, the protein SAND-1 (Mon1-a/b in human) appears to function as an effector that modulates Rab7 recruitment to early phagosomes via Ccz-1 and contribute to nucleotide exchange and Rab7 activation (Poteryaev D et al, 2010; Kinchen JM and Ravichandran KS, 2010). Nucleotide exchange and activation of Rab7 are mediated by HOPS complex that is required for the endosome acquisition of Rab7 and, presumably for Rab5 to Rab7 exchange on phagosomes (Rink J et al, 2005; Poteryaev D et al, 2010; Kinchen JM and Ravichandran KS, 2010).

Once Rab7 is acquired it mediates completion of late phagosomes maturation allowing recruitment of lysosomes to the vacuolar membrane (Bucci C et al, 2000*;* Harrison RE et al, *2003*). Although Rab7 is vital for the completion of phagosome maturation, few of its effectors have been identified. Two such effectors are Rab7-interacting lysosomal protein (RILP) and oxysterol-binding protein–related protein 1 (ORPL1). They accumulate in a Rab7-dependent manner on late degradative endosomes, phagosomes and lysosomes. Together RILP and ORPL1 coordinate microtubule-dependent vesicular traffic of Rab7-positive compartments through direct association with the molecular motor dynein/dynactin. Although necessary, Rab7 and its effectors are insufficient to mediate phagosome/lysosome fusion as demonstrated by the finding that PI3K antagonists block phagosome maturation but do not eliminate Rab7 recruitment and activation (Cantalupo G et al, 2001; Harrison RE et al, 2003; Vieira OV et al, 2003; Johansson M et al, 2007).

LAMP-1 and -2 are also required for late phagosome maturation. They are heavily glycosylated integral membrane proteins that are abundant in late endosomes, lysosomes and mature phagosomes. Although thought to be involved in the preservation of the lysosomal membrane integrity, it has been reported that they are essential for Rab7 recruitment and for the acquisition of microbicidal functions by phagosomes (BinkerMG et al, 2007; Huynh KK et al, 2007).

Retrieval and disposal of membrane components via recycling and MVB biogenesis also occur at this stage of maturation. Indeed similarly to late endosomes, late phagosomes contain

lysobisphosphatidic acid (LBPA), unique lipid found in ILVs of MVBs, and the tetraspanin protein CD63a (Flannagan RS et al, 2009).

The phagolysosome

Once the appropriate material has been dispersed from the phagosomes either to the *trans*-Golgi Network (TGN) via recycling or to the phagosome lumen by ILV formation, the late phagosomes proceed further along the maturation pathway by fusing with preformed lysosomes to form the phagolysosomes through a Rab7 dependent process. The phagolysosome is the ultimate organelle able to kill microrganisms and to digest proteins, lipids, and carbohydrates. The phagolysosome can be differentiated from late phagosome by its marked acidity (pH 4.5–5.0) and the absence of PI(3)P, mannose-6-phosphate receptors (M6PR), and LBPA. In addition, it acquires a sophisticated arsenal of antimicrobial and hydrolytic effectors to eliminate and degrade the engulfed microorganisms.

Phagolysosome acidification not only contributes to degradation, but it is also required for the activation of lysosomal hydrolases, such as cathepsins, that function optimally at low pH.

The destructive capacity of the phagolysosomes arises from the concerted activity of numerous effectors, including hydrolytic enzymes, oxidants and cationic peptides. However, the relative contribution of the individual lytic mechanisms varies among the different types of phagocytes depending on their primary function: for instance pathogen eradication in the case of neutrophils or antigen presentation for DCs (Flannagan RS et al, 2009; Flannagan RS et al, 2012).

Microbicidal mechanisms of the phagosome

Phagosome acidification

The acidification of the phagosomal lumen is generated by the vacuolar V-ATPases. Mature phagosomes have a reduced passive proton permeability that enables accumulation of the pumped protons by the V-ATPases. The V-ATPase is a multimeric protein complex (≈ 103-kDa) consisting of two major functional subcomplexes, the V1 and the V0. The cytosolic V1 hydrolyzes ATP and transfers the energy to the membrane-embedded V0 complex. V0 constitutes the pore through which H^+ are translocated across the bilayer into the lumen of the phagosome at the expense of ATP (Beyenbach KW and Wieczorek H, 2006; Marshansky V and Futai M, 2008).

The extremely acidic luminal pH generated by the V-ATPase requires other ion-transport processes to prevent the buildup of a prohibitive electrical potential across the phagosomal membrane. The inward anions flux (mainly Cl^-), as well as the efflux of cations (probably K^+ and Na^+) neutralize the electrogenic effect of the V-ATPase (Graves AR et al, 2008; Steinberg BE et al, 2010).

Phagosomal acidification has a key role in the phagosome physiology. The acidity directly restricts microbial growth and indirectly activates many hydrolytic enzymes, such as cathepsins D and L, enhancing the degradative capacity of the phagosomes. In addition, the proton gradient across the

phagosome membrane stimulates the natural resistance–associated macrophage protein 1 (NRAMP), which extrudes essential microbial nutrients from the phagosomal lumen. The V-ATPase also participates in the generation of reactive oxygen species (ROS). Indeed, superoxide anions (O_2^-) generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase combine with H^+ in the lumen of the phagosome, generating more-complex ROS (Beyenbach KW and Wieczorek H, 2006; Winterbourn CC, 2008; DeCoursey TE, 2010).

Phagosome acidification appears also to be required to complete the maturation process, as it directly controls membrane trafficking and the recruitment of some protein complex.

Finally, the magnitude of the H^+ gradient across the phagosome membrane can vary greatly depending on the phagocyte type and its role. In particular macrophages, which are effective antimicrobial cells, fully acidify their phagosomes. In contrast the phagosomes of DCs, whose primary function is to present antigens, are considerably more alkaline (pH ≈ 6.0) (Flannagan RS et al, 2009; Flannagan RS et al, 2012).

Reactive oxygen and nitrogen species

ROS are a group of highly reactive free radical and non-radical molecules (Rada B et al, 2008). Although ROS can be generated by different cellular sources, including xanthine oxidase, peroxisomes, the mitochondrial and electron transport chain, the NADPH oxidases (NOXs) represent the predominant in humans. The NOX family comprises seven members (NOX 1-5 and DUOX 1-2) of which NOX2 isoform is the main producer of ROS in phagocytic cells (Nauseef WM, 2004). Because ROS production is most prominent in neutrophils most of the knowledge of NOX2 biology is derived from this cell type. The importance of NOX2-derived ROS in immune cells was clearly demonstrated by genetic studies in late 1980s on patients suffering from chronic granulomatous disease (CGD). These studies showed that CGD patients are susceptible to fungal and bacterial infections due to mutations in the NOX2 gene (Segal AW, 1996; Flannagan RS et al, 2009). NOX2 activity is among the earliest and most robust defense that phagocytes activate against microbes. The NOX2 is a multimeric protein composed of the integral membrane proteins gp91phox and gp22phox (also known collectively as the cytochrome b558) and four regulatory cytosolic subunits p40phox, p47phox, p67phox, and the small GTPase Rac2.

In the absence of proinflammatory stimuli, the cytochrome b558 resides in intracellular vesicles, while cytosolic Rac2 remains inactive via interaction with RhoGDI. Upon the initiation of phagocytosis, GDP-Rac2 is activated by a Rac guanine nucleotide exchange factor and translocates to the plasma or phagosomal membrane, where it allows the transit of cytochrome b558 from the vesicle to the membrane. Concurrently p47phox is phosphorylated and interacts with p22phox. Furthermore p47phox also binds to (PI(3)P) and PI(2)P, generated at the plasma membrane upon

phagocytosis, further stabilizing its localization to cytochrome b558. Several kinases could be responsible for p47phox activation, such as protein kinase C (PKC), protein kinase A, p21 activated kinase , ERK1/2, AKT PI3K and possibly others (Lam GY et al, 2010).

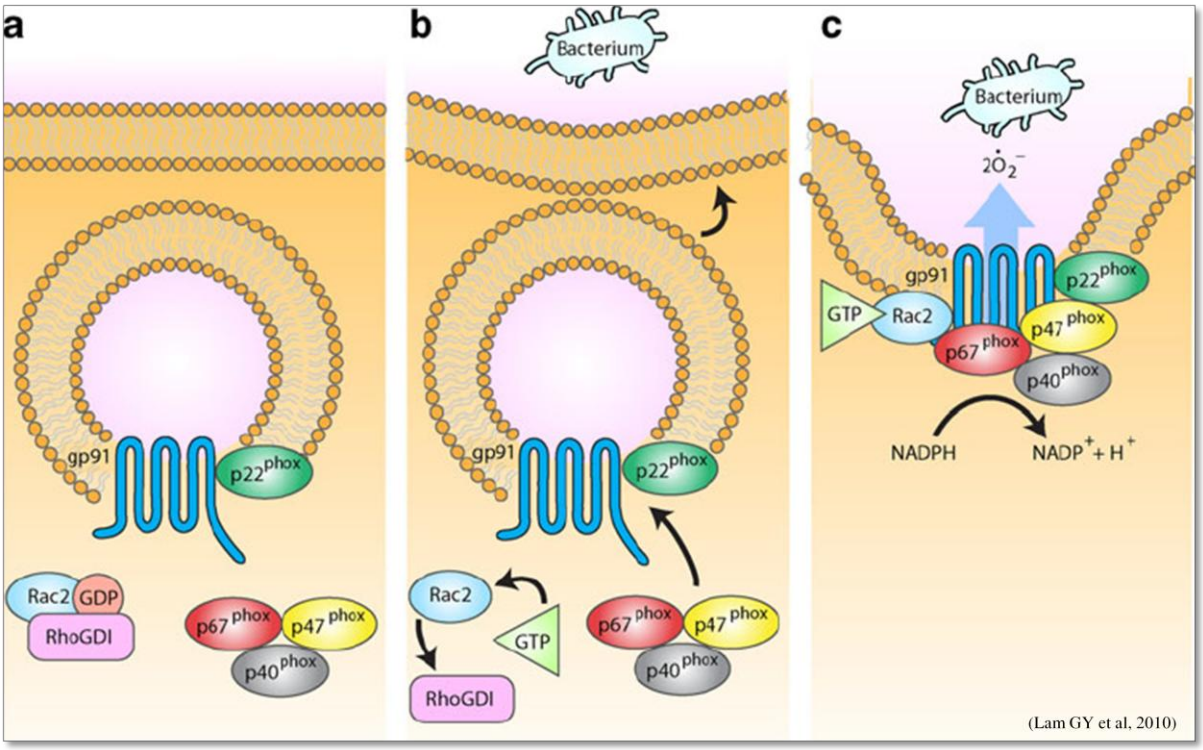


Figure 8. *NOX2 NADPH oxidase assembly and activation. a) In the resting stage, cytochrome b558 (gp91phox and p22phox) resides in vesicles. Rac2 in the inactive GDP bound form remains in the cytosol. The regulatory subunits, p47phox, p67phox, and p40phox, are trimerized in the cytosol. b) Upon receiving signals for activation, cytochrome b558 and the trimeric regulatory subunits are recruited to the membrane. RhoGDI inhibition of Rac2 is now released to allow GTP binding. c) The assembled complex functions at the membrane.*

Since p47phox, p67phox and p40phox are trimerized in the cytosol, the translocation of p47phox brings the other two regulatory subunits to the membrane (Lapouge K et al, 2002).

After assembly and activation NOX2 produces ROS by a reaction on the cytoplasmic region of the gp91phox subunit that converts NADPH to $NADP^+$, resulting in the liberation of two electrons and one H^+. The two electrons are transported through cytochrome b558 to the lumen of the phagosome where they react with two oxygen molecules to form two superoxide ions (O^{-2}). O^{-2} itself is a cytotoxic species, but due to its highly unstable nature it readily forms other compounds (Lam GY et al, 2010).

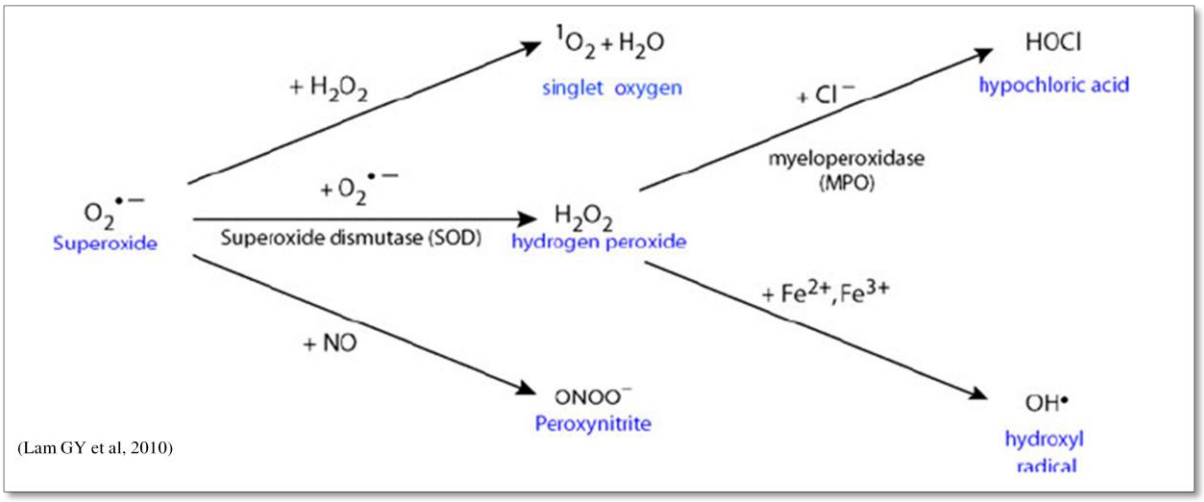


Figure 9. *ROS.*

O^{-2} reacts with nitric oxide to produce peroxynitrite, an even stronger oxidizing agent. Two molecules of ions O^{-2} can be dismutated to form the highly reactive hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). H_2O_2 acts mainly upon thiol groups in cysteine residues, leading to either oxidation or disulfide bond formation. H_2O_2, in turn, interacts with transition metal ions, such as ferrous and ferric ions, to produce hydroxyl radicals (OH•), or with superoxides to generate singlet oxygen ($^1O_2$). In particular OH•, while short lived, is the most highly oxidizing member of the ROS family, reacting rapidly and non-discriminatorily with DNA, lipids and proteins. Moreover the enzyme myeloperoxidase utilizes H_2O_2 to convert chloride ions into deadly hypochlorous acid (Rhee SG et al, 2000; Georgiou G, 2002; Cho SH et al, 2004; Biswas S et al, 2006).

Beside ROS, reactive nitrogen species (RNS) play an important antimicrobial effect into the phagosomes contributing to pathogen eradication by non-selective damage of proteins, lipids and nucleic acids. RNS production is prominent in macrophages in which they have been studied in greatest detail. Their synthesis in phagocytes is catalyzed by inducible nitric oxide synthase (iNOS2). RNS production requires *de novo* synthesis of the iNOS in response to proinflammatory agonists in contrast to ROS, which are produced through a rapid assembly of the preexisting components of the NADPH oxidase. Induction of NOS2 expression requires proinflammatory cytokines (e.g. TNF-α) and/or PAMPs, such as LPS and lipoteichoic acid (DeCoursey TE, 2004; Flannagan RS et al, 2009; Pautz A et al, 2010; Flannagan RS et al, 2012). Functionally, NOS2 operates as a homodimer that consists of an N-terminal oxygenase domain and of a C-terminal reductase domain (Aktan F, 2004). The C-terminal domain transfers electrons from NADPH to flavin adenine dinucleotide (FAD), then to flavin mononucleotide (FMN). The N-terminal domain, that binds the substrate L-arginine, haem, and tetrahydrobiopterin (BH4), produces NO• and citrulline from l-arginine and oxygen using the electrons generated by the adjacent reductase

domain. NOS2 requires the essential cofactor (BH4) for homodimerization and its activity depends on the availability of arginine (Pautz A et al, 2010).

Unlike O_{-2}, NO• is synthesized in the cytosolic face of the phagosomal membrane and subsequently it diffuses into the phagosome lumen. Although NO· is itself a toxic radical, in the luminal environment it readily reacts with ROS undergoing spontaneous or catalytic conversion to a variety of RNS, including nitrogen dioxide (NO2•), peroxynitrite ($ONOO_{-}$), dinitrogen trioxide (N_2O_3), dinitrosyl iron complexes, nitrosothiols and nitroxyl (HNO) (Fang FC, 2004).

In summary, ROS and RNS synergize to exert highly toxic effects to intraphagosomal microorganisms by reacting with a plethora of proteins and nucleic acids.

Antimicrobial proteins and peptides

Phagocytes display a great array of proteins and peptides that antagonize microbial survival into the phagosomes (Skaar EP, 2010). These have been investigated in more detail in neutrophils because they possess specialized secretory organelles (granules) that contain a broad spectrum of bactericidal and degradative proteins, some of which are also expressed by macrophages. Phagocytic antimicrobial factors are grossly subdivided into those that prevent the growth and those that compromise the integrity of the microorganisms.

Growth prevention can be accomplished by limiting the availability of essential nutrients inside the phagosome. To this end, phagocytes secrete scavengers into the lumen or insert transporters into the phagosome membrane. NRAMP1 (also known as SlC11A1) is an integral membrane protein expressed in late endosomes and lysosomes that is recruited to the phagosome membrane soon after pathogens engulfment. NRAMP1 utilizes the electrochemical proton gradient generated by the V-ATPase to extrude from the phagosome lumen divalent cations, such as Fe^{2+}, Zn^{2+} and Mn^{2+}, restricting bacterial growth. Indeed Fe^{2+} and Zn^{2+} are cofactors of many microbial housekeeping enzymes and Mn^{2+} is required by superoxide dismutase, a key protective enzyme expressed by certain pathogens (Searle S et al, 1998; Cellier MF et al, 2007). Fe^{3+} is also sequestered by the scavenger protein lactoferrin. In neutrophils this glycoprotein is stored within granules and it is released into the phagosomes following the fusion of the two organelles. Beside its scavenger role, lactoferrin also impairs pathogen survival through direct interaction with the microbial cell surface (Jenssen H and Hancock REW, 2009).

Phagocytes also dispaly a plethora of antimicrobial proteins and pepetides that affect the **integrity of the microorganisms** reacting directly with membranes, proteins or carbohydrates of the ingested pathogens. Lysozyme hydrolyzes β(1-4) glycosidic linkages between the main structural components of the bacterial peptidoglycan layer, compromising the integrity of Gram-positive bacteria while Gram-negative organisms are partially protected from lysozyme-mediated attack by

their outer membrane (Flannagan RS et al, 2012). However phagocytes also express cationic antimicrobial peptides (CAPs), defensins and cathelicidins, which have a wide range of microbicidal activities against Gram-positive as well as Gram-negative bacteria. These peptides share some common features including a large number of positively charged residues and the ability to assume amphiphilic conformations such as α-helices or β-sheets (Rogan MP et al, 2006).

The defensins, subdivided into α and β subgroups, are small disulphide-bridged polypeptides of 3- to 5-kDa that in neutrophils are stored within azurophil (primary) granules. They bind electrostatically to negatively charged bacterial cell-surface components and exert their microbicidal effect by forming multimeric ion-permeable channels in the mebrane of Gram-positive and Gram-negative bacteria, which allow the diffusion of ions across the bacterial membrane dissipating essential gradients and creating an osmotic imbalance (Risso A, 2000.).

The cathelicidins are also small proteins of ≈ 10 kDa, in neutrophils stored as proforms in the secondary granules (Zanetti, M, 2005). In contrast to the multiple defensins only one cathelicidin gene, *CAMP*, has been found in humans (Sorensen OE et al, 1997; Agerberth B et al, 2000). The gene product of the human cationic antimicrobial (hCAP18) is synthesized as a propeptide that is then cleaved into cathelin and the C-terminal LL-37, the latter exhibiting a broad antimicrobial activity (Sorensen OE et al, 2001). The precursors are converted to active species by elastase, a primary granule protein they probably encounter in the phagosomal lumen. Cathelicidins act by permeabilizing the cell wall and inner membrane of Gram-positive bacteria and the outer and inner membranes of Gram-negative bacteria (Zanetti M, 2005).

Finally, more than 50 different lysosome acid hydrolases are required for the degradative capacity of the phagolysosome. Indeed the concerted action of proteases, lipases, nucleases, glycosidases and phosphatases mediates the complete destruction of complex structures, such as dead or dying microbes previously processed by the phagocyte antimicrobial factors.

Cathepsins are a family of lysosomal enzymes that comprises serine proteases (cathepsins A and G), aspartate proteases (cathepsins D and E) and cysteine proteases (cathepsins B, C, F, H, K, L, O, S, V, X, and W). Cysteine proteases possess both endo- and exopeptidase activities, whereas aspartate proteases are endopeptidases and serine proteases exopeptidases only. Endopeptidases, particularly the C1 family of cysteine proteases, are particularly important because they efficiently generate substrates for the exopeptidases (Pillay CS et al, 2002). Lysosomal cathepsins are synthesized as pre-proenzymes and processed to the active forms in the late endosomes. The cleavage of the N-terminal signal peptide occurs during the passage to the endoplasmic reticulum in parallel with the N-linked glycosylation. After the removal of the signal peptide, the propeptides are transported to the endosomes/ lysosomes via the mannose-6-phosphate receptor pathway (M6PR),

and they also act as inhibitors to prevent any inappropriate proteolytic activity of the zymogen (Hasilik A et al, 2009; SaftigP and Klumperman J, 2009; Schroder BA et al, 2010). In the mildly acidic environment of the endosomes the N-terminal propeptide is removed either autocatalytically or by other proteases. The mature proteolytically active cathepsins are released to lysosomes either through endosome maturation or through endosome-lysosome fusion (Repnik U et al, 2012). Cathepsins may be activated or inhibited inside the phagosomes, While acidity activates these enzymes, the redox state of the phagolysosomes imposed by the NADPH oxidase diminishes their proteolytic activity. This complex regulation may be needed to ensure that proteases are fully active only when the time is right, such as cathepsin H and S that are delivered to early and late phagosomes, respectively. Indeed not all the proteases are acquired simultaneously by the maturing phagosomes, implying that they are delivered by distinct organelles. Beside the peptidases, hydrolases targeting carbohydrates (for example, α-hexosaminidase, β-glucuronidase and lysozyme) and lipids (for example, phospholipase A2) also act in the phagosomes (Flannagan RS et al, 2009; Pautz A et al, 2010; Flannagan RS et al, 2012).

Pulmonary macrophages

Lungs are continuously exposed to environmental microbes and particulate materials, representing the major sites of primary viral and bacterial infections (Martin TR and Frevert CW 2005; Ratjen F et al, 2012). In the lung, which comprises parenchyma and alveolar space, several factors cooperate to maintain the homeostasis. Particles that are carried into the conducting airways encounter soluble constituents in airway fluids and the upward propulsive force of the mucociliary system. Particles that cannot be cleared by the mucociliary movement reach alveolar surface where they interact with the alveolar fluids (e.g. IgG, complement, surfactant and surfactant-associated proteins) and alveolar macrophages (Martin TR and Frevert CW, 2005). The macrophages can be categorized in different types based on their localization in the lung: the alveolar macrophages, the parenchymal (lung) macrophages and the intravascular macrophages. In steady state condition alveolar macrophages represent approximately more than 90% of the immune cells in the lung and due to their location, at the interface between the air and lung tissue (Peters-Golden M, 2004), they represent the first line of defence to inhaled pathogens and environmental particles (Chaudhuri N and Sabroe I, 2008). Nevertheless, the primary roles of the alveolar macrophage is to keep the airspaces clean by efficient removal of inert inhaled particles (like amorphous silicates and carbongraphite) without triggering an inflammatory responses that could damage the alveolar type I and type II cells (Martin TR and Frevert CW, 2005; Sulahian, T. H. et al, 2008). Several suppressive pathways act in combination to limit their responsiveness to external stimuli. They show lower levels of MHC class II and co-stimulatory molecules than their tissue-resident

counterparts (Steinmuller C et al, 2000) being no efficient as antigen-presenting cells (APC) and poor T cell stimulators (Kuolee R and Chen W, 2008). In addition they produce low amounts of inflammatory cytokines and actively inhibit T cell response via secretion of prostaglandins, transforming growth factor beta (TGF-β) and NO (Sorio C and Melotti P, 2009).

Interactions of alveolar macrophages with other cells in the airways, such as DCs, may also limit inflammatory responses in the steady-state and, reciprocally, the DCs exposed to antigens in the airway lumen produce IL-10, a broadly inhibitory cytokine which further limits the local inflammatory response. In the presence of infection, alveolar macrophages following the interaction with bacteria profoundly change their functions. Once activated, alveolar macrophages generally display a greater phagocytic capacity, higher oxidative burst and pro-inflammatory cytokine production than resting cells. In more details, macrophages produces proinflammatory cytokines such as: GM-CSF and TNF-α that overcome the inhibition of T cells responses; IL-8 and the related CXC chemokines that initiate a localized inflammatory response by recruiting neutrophils from the lung capillary networks into the alveolar space (Lohmann-Matthes ML et al, 1994; Steinmuller C et al , 2000; Martin TR and Frevert CW, 2005). Ultimately they are also responsible for clearing apoptotic neutrophils from the site of infection by phagocytosis, thus avoiding chronic inflammation.

The lung or parenchymal macrophages are located in the lung connective tissue, and seem to have intermediate functional properties, both as cells of second line defence and APC. Compared to alveolar macrophages, they show a reduced production of TNF-α and oxygen radicals whereas their accessory function and MHC class II expression are increased.

Finally, intravascular macrophages are located on the endothelial cells of the capillaries facing the bloodstream and are believed to remove foreign and damaging material which enters the lung via the bloodstream (Lohmann-Matthes ML et al, 1994).

Pulmonary macrophages have been reported to be derived both from precursors in peripheral blood and from proliferation of local precursors. Although it is assumed that a monocyte subset might be the precursor of alveolar macrophages, this has not been directly tested. Nevertheless, Landsman L et al 2007 reported that alveolar macrophages originate from blood monocytes trough a lung-resident intermediate. According to this model, production of alveolar macrophages involves the differentiation of blood monocytes into macrophages in the lung parenchyma, proliferative expansion of these cells and their migration into the alveolar space. In such scenario, lung macrophages may serve as a local reservoir from which alveolar macrophages can be generated whenever needed (Landsman L and Jung S, 2007; Gordon S and Taylor PR, 2005).

Macrophages in cystic fibrosis

CF macrophages display differences in number and phenotype compared to non-CF macrophages. First of all, the number of alveolar macrophages in young non-infected CF patients has been reported to be elevated compared to non-CF individuals (Brennan S et al, 2009) suggesting a constitutive/intrinsic and early mononuclear inflammation in CF. The expression of scavenger receptors, such as macrophage receptor with collagenous structure (MARCO) and CD206 (mannose receptor), has also been reported to be reduced in CF macrophages probably contributing to the impaired clearance of inhaled particles and apoptotic cells as well as to increased inflammation and damage in CF lungs (Rao S et al, 2009).

Macrophages polarization in CF patients is controversial, indeed despite some attempts to study this aspect the results are inconclusive so far: for instance circulating CF monocytes and macrophages isolated from BAL from *P. aeruginosa* infected CF patients exhibited a M2 phenotype compared to the controls, while macrophages isolated from CF nasal polyp explants showed a M1 profile (del Fresno C et al, 2009; Murphy BS et al, 2010; Krysko O et al, 2009). All these data lead to the conclusion that macrophage phenotype is altered in CF. The question whether macrophage alterations are the result of exposure to the CF lung environment, just reflecting the inflammation and the infection, or whether CF macrophages display an intrinsic/autonomous defect, directly contributing to the pathogenesis of CF lung disease, is not fully elucidated. Nevertheless recent studies provide experimental evidence supporting the notion that immune cells actively contribute to CF-related lung disease.

The expression of CFTR in non-epithelial tissues has been described (Yoshimura K et al, 1991). Particularly CFTR protein is detectable in both murine (Deriy LV et al, 2009; Radtke AL et al, 2011) and human macrophages (Del Porto et al 2011; Sorio C et al, 2011) and the CFTR-like Cl^- conductance has been recorded in monocytes/macrophages (Di A et al, 2006; Radtke AL et al, 2011; Del Porto et al 2011; Bruscia EM et al. 2011), suggesting that in these cells CFTR functions as a cAMP-dependent chloride channel.

Accordingly, it has been shown that CF macrophages fail to perform their functions properly. Murine CF alveolar but not peritoneal macrophages show an impaired microbicidal activity, possibly due to defective phagolysosome acidification suggesting that CFTR contributes to phagosomal pH control. Di and colleagues demonstrated that alveolar macrophages from *Cftr–/–* mice although retaining the ability to phagocytose and to generate an oxidative burst, were defective in bacterial killing. Measurements of lysosomal pH in C*ftr–/–*mice as well as in ΔF508 and G551D CFTR mutant mice demonstrated that this organelle did not acidify properly. Thus the

authors speculated that CFTR contributes to lysosomal acidification thus providing an environment conducive to bacterial replication (Di A et al, 2006; Deriy LV et al, 2009).

In a more complex scenario, pH alteration in intracellular vesicles has been correlated to the impaired activity of the acid sphingomyelinase, a pH-sensitive enzyme involved in ceramide metabolism. Ceramide forms large ceramide-enriched membrane platforms required for diverse signal transductions. Recent studies indicate that ceramide play an important role in the outcome of infections. It has been reported that ceramide accumulates in an age dependent manner in the respiratory tract of uninfected CF mice and in human CF macrophages. It has been proposed that, alkalinization of the intracellular vesicles, causes an imbalance between acid sphingomyelinase (Asm) cleavage of sphingomyelin to ceramide and acid ceramidase consumption of ceramide, resulting in the higher levels of ceramide thus favoring the establishment of the infectious-inflammatory vicious cycle typical of CF lung disease (Teichgräber V et al, 2008).

More recently, the same research group correlated the impaired ceramide metabolism to the redox signaling that regulates alveolar macrophage apoptosis upon infection with *P.aeruginosa* (Zhang Y et al, 2008). Under physiological conditions, following *P. aeruginosa* infection, alveolar macrophages through the formation of ceramide-enriched membrane platforms mediate the assembly and activation of the NADPH oxidase leading to ROS production which, in turn, contributed to *P. aeruginosa* killing. In CF alveolar macrophages, ceramide has been shown to accumulate in vesicles, presumably secretory lysosomes, which exhibit a higher pH compared to that of corresponding vesicles isolated from wild type alveolar macrophages. Alkalinization of these vesicles does not allow Asm and acid ceramidase to function properly thus leading to ceramide accumulation. Based on these data Zhang Y et al. proposed a model in which CFTR deficiency results in chronic accumulation of ceramide in macrophages but prevents its acute release following *P. aeruginosa* infection. This defect result in a failure to assemble and activate NADPH oxidase and which in turn compromise ROS release and *P. aeruginosa* killing (Zhang Y et al, 2010).

Other works suggest that the phagosome Cl^- concentration can modulate intracellular bacteria viability. For instance, the CFTR-mediated Cl^- flux in macrophages was proposed to contribute to the *Listeria monocytogenes* phagosomal escape to the host cytosol by favoring its hemolytic activity (Radtke AL et al, 2011). In addition, the defective CFTR-mediated Cl^- flux in CF macrophages was found to impair autophagy and autophagosome formation during Burkholderia infection. CF mouse macrophages infected with Burkholderia downregulated the expression of autophagy-related genes compared to WT cells, impairing the autophagosome–lysosome fusion and thus reducing bacterial clearance (Abdulrahman BA et al, 2011).

One important aspect of macrophages physiology is their response to bacterial stimuli by secretion of cytokines and chemokines that contribute to inflammation. In CF it has been reported that macrophages contribute significantly to the hyperinflammatory response to bacterial stimuli. Indeed, LPS-stimulated mouse CF macrophages secrete higher levels of IL-1a IL-6, G-CSF and IL-8 compared to WT cells (Bruscia EM et al, 2009). Moreover, it appears that CFTR in macrophages influences TLR4 spatial and temporal localization that is important to control LPS-mediated signaling. Upon LPS stimulation CFTR macrophages shown prolonged TLR4 retention in the early endosome and reduced translocation into the lysosomal compartment. This abnormal TLR4 trafficking led to increased LPS-induced activation of the NF-kB, MAPK, and IFN regulatory factor-3 pathways and decreased TLR4 degradation, which affects the downregulation of the proinflammatory state. Thus, the abnormal trafficking of TLR4 probably contributes to the increased secretion of pro-inflammatory cytokines in CF macrophages (Bruscia EM et L, 2011).

Finally, CF macrophages have been described to be defective in neutrophils removal by apoptosis (efferocytosis) thus favoring neutrophilic inflammation in CF (Hartl D et al, 2012).

Taken together these observations suggest that CF macrophages display several intrinsic abnormalities that alter their ability to properly control inflammatory response and to kill bacteria in the context of CF lung disease. In the early stages of CF lung disease, the inappropriate macrophages behavior may contribute to conditions that favor bacterial lung adaptation, harmful chronic infection and lung damage.

Matrix metalloproteinases (MMPs)

MMPs are one of four subfamilies within the superfamily of metalloendopeptidases Zn^{2+}-dependent metzincins (Stocker W and Bode W, 1995). More than 20 MMPs have been identified, and further subdivided into collagenases, gelatinases, elastases and stromelysins based on their structure and substrate specificity, nevertheless their substrate specificity often overlap. All MMPs exhibit the same modular structure, that include from N- to C-terminus: the signal sequence (or pre-domain) zymogenic pro-peptide (pro-domain), an active catalytic domain (with a zinc-binding region) and hemopexin-like domain (Tallant C et al, 2010). The pre-domain, indispensible for secretion, is removed after it directs their synthesis to the endoplasmic reticulum; the ~80-residue pro-domain maintains enzyme latency until it is removed or disrupted (Nagase H and Woessner JF, 1999); the ~165-residue zinc- and calcium-dependent catalytic domain dictates cleavage-site specificity (Overall CM, 2001; Tallant C et al, 2010) and the hemopexin-like domain works for collagen binding, pro-MMP activation, and dimerization (Gomis-Rüth FX, 2004; Masko K, 2005). Although all MMPs share this modular combination, specific domains are inserted in some MMP.

MMPs are transcriptionally regulated: various cytokines, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α, induce MMP transcription through the activation of different intracellular signaling cascades (Gaggar A et al, 2011). In addition to transcription-level regulation, most MMPs are translated as zymogen inactive form. The pro-enzymes are secreted and subsequently activated (either by another protease or auto-activation by the active form of that MMP) in the extracellular space where MMPs are thought to perform their biological functions. Inhibitors of MMPs represent a further level of regulation for the activity of these enzymes. Although tissue inhibitors of metalloproteases (TIMPs) are the most specific endogenous MMP inhibitors, the inhibition *in vivo* also occurs through relatively nonspecific inhibitors such as α-2 macroglobulin (Sternlicht MD and Werb Z, 2001; Stamenkovic I, 2003).

MMPs perform numerous biological functions, including degradation of matrix components and remodelling of tissues, release of cytokines, growth factors and chemokines, and modulation of cell mobility and migration. Due to their involvement in many normal homeostatic mechanisms, when aberrantly or excessively expressed, MMPs cause tissue destruction contributing to different pathological conditions, such as emphysema, vascular disease and arthritis (Houghton AM et al, 2009).

Although virtually all MMP functions had been thought to occur outside the cell or on the cell surface, MMPs have recently been implicated in intracellular functions including apoptosis and cell cycle regulation (Golubkov VS et al, 2005; Limb GA et al, 2005). Moreover it has been reported that MMP, as well as other proteinases, including cathepsin G, proteinase3 and neutrophil elastase,

possess direct antimicrobial activity, independent of their catalytic activity (Belaaouaj A et al, 1998; Belaaouaj A et al, 2000; Weinrauch Y et al, 2004).

Monocytes/macrophages produce MMPs, such as MMP-1 (interstitial collagenase), MMP-2 (type IV, 72-kDa collagenase), MMP-9 (92-kDa collagenase), MMP-3 (stromelysin 1), MMP-10 (stromelysin 2), MMP-7 (matrilysin) and MMP-12 (O'Connor C and FitzGerald M, 1994; Shapiro S, 1994). Although the molecular mechanisms regulating MMPs production are incompletely defined, previous studies indicated that the spectrum of MMPs produced and their regulation vary during monocyte/macrophage differentiation (Welgus HG et al, 1990; Campbell EJ et al, 1991).

MMP in cystic fibrosis

Macrophage MMPs principally involved in CF are MMP-2, MMP-9 and MMP-12 (Table2). Gelatinases, a key subgroup of MMPs, include gelatinase A and gelatinase B which also called matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), respectively.

These proteinases can specifically degrade native collagens and/or basal membrane proteins (Momota Y et al, 2005; Duran-Vilaregut J et al, 2011; Zhou TB and Qin YH, 2012). MMP-2 is expressed constitutively in various cell types, it is activated in membrane-type MMP-dependent manner and is involved in modulating diverse cellular functions, including angiogenesis, tissue remodelling and potentiation of inflammatory response. Although not extensively studied, the potential role of MMP-2 in CF lung disease has been hypothesized (Ward RV et al, 1991; Kumagai K et al, 1999; Brown MD and Hudlicka O, 2003; Chakrabarti S and Patel KD, 2005; Page-McCaw A et al, 2007; Gaggar A et al, 2011).

MMP-9, one of the most extensively studied proteases, is also broadly expressed in a variety of cells including inflammatory PMNs and macrophages. MMP-9 plays different functions, some of which appear to have opposite effects. For example, MMP-9 has been involved in the inflammatory response by inducing the production of the proline-glycine-proline (PGP) peptide (Gaggar A, et al, 2008) and the chemokine potency of IL-8 (Van den Steen PE et al, 2000). However, it is also involved in the regulation of granuloma formation in tuberculosis (Volkman HE et al, 2010). MMP-9 was found to increase in the lower airway secretions of CF patients (Ratjen F et al, 2002; Gaggar A et al, 2007). Differently from the other MMPs, MMP-12, also known as macrophage elastase, is exclusively secreted by macrophages and is predominantly expressed in mature tissue macrophages. This enzyme, in addition to elastin, degrades a broad spectrum of substrates, including type IV collagen, fibronectin, laminin, vitronectin, proteoglycans, chondroitin sulfate, myelin basic protein, δ-1-antitrypsin and plasminogen (Chandler S et al, 1996; Gronski TJ et al, 1997). Another important function of MMP-12 is to activate MMP-2 and MMP-3, which leads to subsequent degradation of other extracellular matrix proteins (Matsumoto S et al, 1998). MMP-12 is secreted

Table 2. *Evidence supporting the involvement of MMPs in CF lung disease.*

	IntEnz number	Alternative name	Molecular weight	Location of expression in airways	Expression in CF	Predicted role in CF
MMP-2	EC 3.4.24.24	Gelatinase A	72 kDa pro 64 kDa active	Structural cells (epithelia, endothelium) and macrophages	Detected in BAL fluid, not detected in sputum, decreased in serum during exacerbation	Regulation of ion transport, increasing remodelling and inflammatory response
MMP-7	EC 3.4.24.23	Matrilysin	28 kDa pro19 kDa active	Epithelial cells predominantly	Elevated in airway epithelia	Possible role in injury/repair
MMP-8	EC 3.4.24.34	Neutrophil collagenase	75 kDa pro 65 kDa active	Neutrophils and epithelia	Elevated in serum and levels negatively correlated with lung function, elevated in BAL fluid	Modulation of inflammatory response, airway remodelling, generation of bioactive collagen fragments
MMP-9	EC 3.4.24.35	Gelatinase B	92 kDa pro 82 kDa active	Predominately neutrophils but also structural cells (epithelia, endothelium) and macrophages	Elevated in lower airway secretions and negatively correlated with lung function, elevated in serum	Modulation of inflammatory response, airway remodelling, generation of bioactive collagen fragments
MMP-12	EC 3.4.24.65	Macrophage elastase	54 kDa pro 45 kDa active	Macrophages	Detected in sputum and serum of CF patients	Possible role in airway and parenchymal remodelling, possible role in generating bioactive elastin fragments

IntEnz: Integrated Enzyme Database; BAL: bronchoalveolar lavage.

(Gaggar A et al, 2011)

from cells as a 54-kDa proenzyme or zymogen consisting of common MMP domains. Upon activation, MMP-12 not only cleaves its prodomain but also has a unique propensity to cleave its C-terminal hemopexin-like domain, resulting in a 45kDa domain and in the 22-kDa catalytic domain (Banda MJ and Werb Z, 1981; Shapiro SD et al, 1993; Wu L et al, 2000). It seems to be subject to partial regulation by proteases, such as thrombin and plasmin, and can be released from macrophages following a variety of inflammatory cytokines stimuli, such as TNF-α and IL-1β. Physiological functions for MMP12 have not been fully elucidated. It has been reported that MMP-12 modulates cytokine and chemokine networks, including cleavage of pro-TNF-α and ELR+ CXC chemokines and it could be also related to remodelling of connective tissue during growth and development, and migration of macrophages into tissues (Churg A et al, 2004; Dean RA et al, 2008; Dasilva AG and Yong VW, 2008). Interestingly, recent data have demonstrated that MMP-12 has direct antimicrobial activity and plays an important role in the macrophage-mediated killing of both Gram-negative and Gram-positive bacteria in the lung and in other organs. In particular Houghton and coworkers demonstrated that, in addition to be secreted as a pro-enzyme, preformed intracellular storage of MMP-12 exists within quiescent macrophages, presumably for rapid secretion from the cells. After engulfment of the microorganisms by macrophages, these preformed stores are mobilized to phagolysosomes, where MMP-12 adheres to bacterial cell and disrupts cellular membranes causing bacterial death (Houghton AM et al, 2009). MMP-12 is implicated in several pathological processes, including emphysema (Shipley JM et al 1996; Hautamaki RD et al, 1997). Recently, increased level of MMP-12 in the sputum of CF patients has been demonstrated (Gaggar A et al, 2007), but its role in CF lung disease has not been fully elucidated. By using the beta-ENaC transgenic mouse model, that recapitulates the CF lung disease, it has been proposed

that airway surface dehydration and muco stasis cause macrophage activation and MMP-12 dependent emphysema (Mall M et al, 2004; Schubert SC et al, 2009) (figure 10).

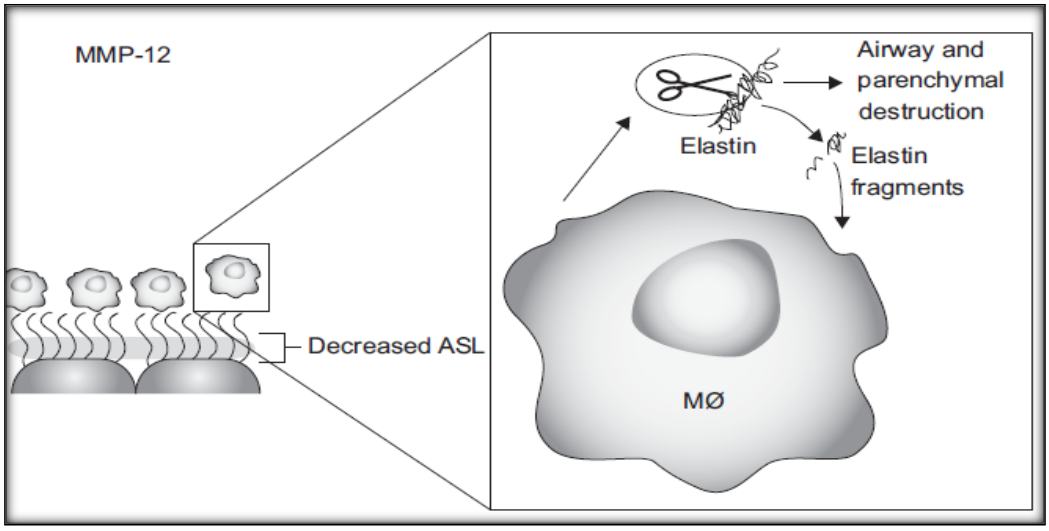


Figure 10. *Predicted effects of MMP-12 in the context of CF.*
According to this model, decreased airway surface liquid (ASL) activates macrophages (MØ) and increases expression of MMP-12, which cleaves elastin, resulting in degradation of the airway and lung parenchyma. These elastin fragments may also increase the recruitment of monocytes and activation of macrophages.

Pseudomonas aeruginosa

Pathogens in CF

Several bacteria species have been detected in CF lungs. During infancy, CF patients are colonized by *Hemaophilus influenzae* and *S. aureus*; however, during adolescence *P. aeruginosa* predominates, being the most common opportunistic pathogen isolated from the respiratory tract of CF patients (figure 11). Other opportunists include nosocomial pathogens such as *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* and species that are only occasionally associated with human infections apart from CF, such as the Bcc, *Ralstonia* species, *Cupriavidus* species, *Klebsiella* species and *Pandoraea* species. Among these bacteria, Bcc represent the most problematic leading to a rapid and progressive deterioration of respiratory function characterized by necrotizing pneumonia, bacteremia and sepsis, the "cepacia syndrome". Beside Bcc infections fungi, such as *Aspergillus* and *Candida* species, are associated with an increased frequency of acute exacerbations in CF patients. CF patients are also susceptible to colonization by *Mycobacterium* species and by anaerobic species belong to the genera *Prevotella*, *Veillonella*, *Proprionibacterium* and *Actinomyces.* Nevertheless, limited information exists on the host–microbial interactions of many of these organisms. Finally, respiratory syncytial virus (RSV) and influenza A and B viruses have been identified in CF patients, but the epidemiology of these viruses among persons with CF does not differ significantly from that of the general population. Thus the impact of viral infections on pulmonary disease in CF remains an active area of investigation (van EwijK BE et al, 2005; Lipuma JJ, 2010).

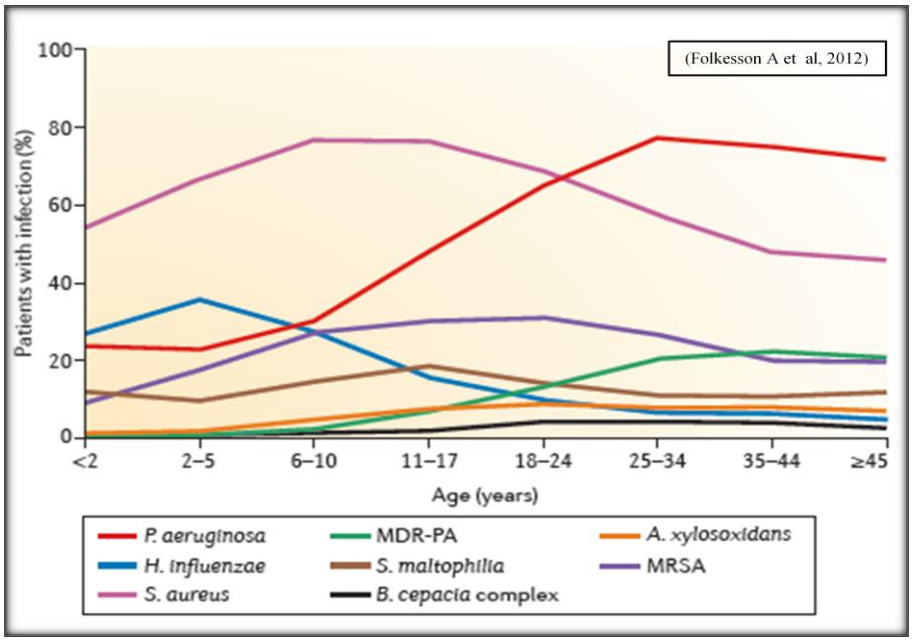


Figure 11. *Prevalences of several common respiratory pathogens in CF as a function of age.*

Pseudomonas aeruginosa

P. aeruginosa is a ubiquitous Gram-negative microorganism found in many environments, such as soil and water. *P. aeruginosa* is also an opportunistic human pathogen able to cause infections in the immunocompromised individuals such as patients with cystic fibrosis (CF), severe burns, or those with impaired immunity as patients infected with HIV or cancer patients undergoing chemotherapy (Wagner VE and Iglewski BH, 2008). This bacterium is the most common pathogen identified in the respiratory secretions of patients with CF, indeed approximately 80% of the adult CF patients are permanently colonized by *P. aeruginosa.* Despite the development of new antibiotics for aerosol therapy, including tobramycin (Lyczak JB et al., 2002; Heijerman H, 2005), *P. aeruginosa* lung infection is the cause of much of the morbidity and most of the mortality in CF patients (Høiby N, 2011). The pathogenesis of *P. aeruginosa* is complex, and the outcome of the infection depends on the virulence factors displayed by the bacteria as well as the host response (Sadikot RT et al, 2005). Multiple bacterial virulence factors of *P. aeruginosa*, and their modulation during the transition from the early to chronic infection, are involved in the persistence of this bacterium in CF airways. The major *P. aeruginosa* virulence factors are reported in table 3.

Table 3. *P. aeruginosa virulence factors.*

Bacterial factor	**Functions**
Pili	Involved in attachment to eukaryotic cells (Sadikot RT et al, 2005)
Flagella	Motility (Sadikot RT et al, 2005).
Type III secretion system	It is activated on contact with eukaryotic cell membranes and interferes with signal transduction, resulting in cell death or alterations in host immune responses (Sadikot RT et al, 2005).
Quorum sensing systems	They coordinate expression of genes important for adaptation to the environment (Sadikot RT et al, 2005).
Pyochelin and Pyoverdin (siderophores)	Providing iron to support bacterial metabolic processes; controlling the expression of other *P. aeruginosa* virulence factors, such as exotoxin A, endoprotease, and pyoverdine itself (Lamont IL et al, 2002).

Elastase	Cleaveage of collagen, IgG, IgA, and complement; disrupting epithelial cell tight junctions and interfering with mucociliary clearance; degradation of surfactant proteins A and D (SP-A and SP-D) (Mariencheck WI et al, 2003).
Alkaline protease	Lysis of fibrin and interfering with its formation; inactivate important host defense proteins, such as antibodies, complement, IFN-, and Cytokines (Sadikot RT et al, 2005).
Leukocidin	pore-forming protein that has cytotoxic effects on host cells (Sadikot RT et al, 2005).
Phospholipase and Lecithinase	hemolysins that act synergistically to break down lipids and lecithin; promotinge invasion by causing cytotoxic effects on host cells (Sadikot RT et al, 2005).
Pyocyanin (N-methyl-1-hydroxyphenazine),	proinflammatory effect; disruption of bronchial epithelium; impairing ciliary function; interfering with the antioxidant defenses in the lung and inhibition of catalase activity (O'Malley YQ et al, 2003).
Exotoxin A	ADP-ribosylating enzyme that enters eukaryotic cells by receptor-mediated endocytosis and catalyzes the ADP-ribosylation of eukaryotic elongation factor-2 inhibiting protein synthesis and ultimately leading to cellular death (Yates SP et al, 2001).

Colonization and chronic infection in CF airways

Most individuals acquire *P. aeruginosa* through casual contact with natural environmental reservoirs (Burns JL et al, 2001). However, transmission of strains among patients can occur, and depend on the frequency of meetings between patients (Folkesson A et al, 2012). Bacteria are thought to first colonize the oropharynx and then enter the lower respiratory tract by microaspiration (Mainz JG et al, 2008). The evolution of P. aeruginosa infection in CF patients is schematically represented in figure 12. Chronic airway infection with *P. aeruginosa* is usually

preceded by a period of recurrent, intermittent colonization of the airways (Johansen HK and Hoiby N, 1992). In this phase, which can last from birth until the patient acquires a chronic infection (usually occurring in their late twenties or early thirties), the infection can be effectively combated with aggressive antibiotic therapy, and this can substantially delay the onset of subsequent chronic infection (Folkesson A et al, 2012). The patient is often re-infected at later time points with different strains rather than to relapse with the same strain (Johansen HK and Hoiby N, 1992; Burns JL et al , 2001), but in approximately 25% of cases re-colonization by the same strain occurs (Folkesson A et al, 2012). The source of the re-colonization may be either a persistent environmental source or an undetected reservoir in the patient (Armstrong DS et al, 1996). The paranasal sinuses potentially constitute one such protected niche from which *P. aeruginosa* can re-colonize the lungs. In effect, patients who are intermittently or chronically infected with *P. aeruginosa* show close agreement between the bacteria residing in their lungs and their sinuses (Folkesson A et al, 2012).

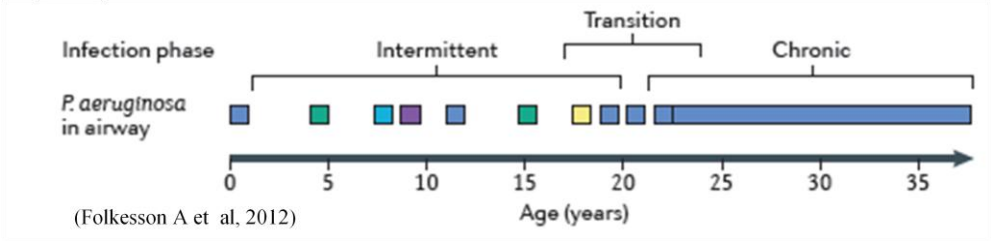


Figure 12. *Schematic representation of the progression of P. aeruginosa infection in a hypothetical patient with CF. Different colours represent phylogenetically independent P. aeruginosa clones. Intermittent colonization can be eradicated, and the patients can be negative for P. aeruginosa for up to several years. The process is repeated until a chronic infection is established.*

At this early stage of infection, the majority of isolates resemble environmental strains in that they are nonmucoid and highly antibiotic susceptible (Burns JL et al , 2001, Speert DP et al, 2002). For unknown reasons, this intermittent colonization phase sooner or later transits into a chronic infection. Once established within the respiratory airways, *P. aeruginosa* resists eradication despite a constant assault by the host immune system and treatment with prolonged courses of antibiotics (Hauser AR et al, 2011).

Chronic infection is characterized by continuous growth of *P. aeruginosa* in airway secretions and by genetic and phenotypic variations of the infecting bacteria (Folkesson A et al, 2012). This phase is characterized by the production of antibodies against *P. aeruginosa*, a higher degree of inflammation, a higher number of infiltrating neutrophils and a greater release of serine proteinases.

Indeed, once bacteria infect the CF airways, bacterial antigens, such as pili, flagella, DNA, and quorum sensing autoinducer molecules (Di Mango E et al, 1995; Delgado MA et al, 2006), are detected by the host and induce release of proinflammatory factors such as IL-8, TNF-α, IL-1, IL-6. In CF airways bacteria, possibly due to their persistence, induce an exaggerated inflammatory response, characterized by a robust recruitment of activated neutrophils to the airway lumen (Hauser AR et al, 2011). This inflammatory cycle, in addition to the damage that is actively caused by the bacteria, leads to lung obstruction and progressive pulmonary injury decreasing lung function (Nichols D et al, 2008).

Evolution of *P. aeruginosa* in chronic infections

To establish a chronic infection in the airways *P. aeruginosa* must survive and adapt to highly stressful conditions that have fundamental effects on its subsequent evolution, such as host immune response, the constant presence of antibiotics, competition with other resident microorganisms, osmotic and oxidative stress (Folkesson A et al, 2012). The mechanisms by which *P. aeruginosa* successfully persists in the CF lungs are unclear but it may involve its impressive ability to adapt to environmental changes and stress conditions figure 13 (Hauser AR et al, 2011).

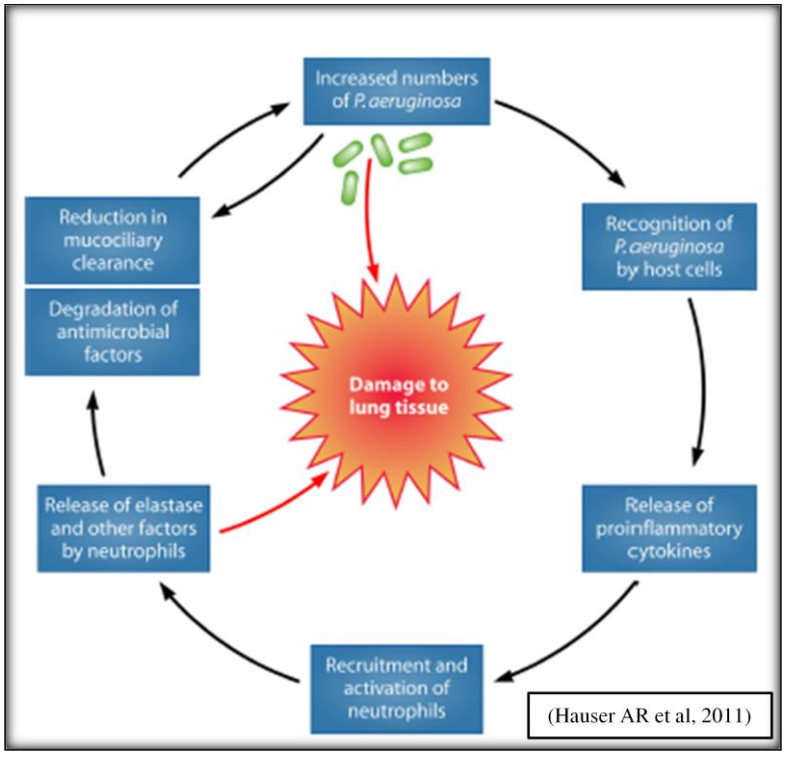


Figure 13. *Cycle by which the presence of P. aeruginosa bacteria in the airways of individuals with CF leads to progressive pulmonary injury.*

During initial infection, the large genome of *P. aeruginosa,* through the modulation of virulence factors, contributes to its ability to persist in the environment.(Wagner VE and Iglewski BH, 2008),

while later, selection of clonal lineages containing spontaneously arising mutations contributes to shape up the bacterial community that colonizes the lung (Smith EE et L, 2006). Spontaneous mutations continuously arise in *P. aeruginosa*, and their rate may increase due to the occurrence of hypermutable strains and by the downregulation of antioxidant enzymes during growth in biofilms (Oliver A et al, 2000; Boles BR et al, 2004; Driffield k ET AL, 2008). These mutations result in the generation of a diverse array of *P. aeruginosa* lineages within the CF airways, many of which exhibit different phenotypes, thus favouring the growth and selection of strains with phenotypic traits that confer an adaptive advantage (Smith EE et al, 2006). Such selection is relatively common in CF but apparently differs in its nature from one portion of the respiratory tract to another, resulting in heterogeneous populations of bacteria that are closely related but possess unique sets of mutated genes (Nguyen D and Singh PK, 2006; Smith EE et al, 2006; Hogardt M et al, 2007).

When *P. aeruginosa* moves from the environment into the CF airways both nutritional and physicochemical changes occur (Folkesson A et al, 2012). Indeed *P. aeruginosa* in the presence of stressful conditions, such as antibiotics or oxidative stress, changes its gene expression profile. Many of the changes are specific for the particular stress, but there is also a common theme that is shared between the responses to several different antibiotics and to environmental stresses such as osmotic shock and magnesium starvation. The switch in gene expression in *P. aeruginosa* is largely governed by the *algU* (also known as *algT*) regulon, which is activated under conditions of cell envelope stress and leads to a coordinated downregulation of central metabolism, motility and virulence and a concurrent upregulation of genes affecting membrane permeability (figure 14) (Aspedon A et al, 2006; Anderson GG et al, 2008; JonesAK et al, 2010). In particular, some adaptive traits that commonly emerge during respiratory infections in CF are the mucoid phenotype, antibiotic resistance, alterations in lipopolysaccharide (LPS), loss of type III secretion system and motility, auxotrophy, (Small-colony variants) SCVs, defects in quorum sensing and hypermutability (Hauser AR et al, 2011).

P. aeruginosa adaptations in CF

P. aeruginosa, and other microorganisms colonizing the CF airways, are subjected to various types of stress. Factors such as antibiotics, the host immune response, and oxidative and osmotic stress are all important evolutionary forces that select for genetic variants with an improved ability to survive and proliferate in the CF-associated environment. Indeed, *P. aeruginosa* strains from chronically infected CF patients are phenotypically different from isolates at the early phase of infection. Many of these phenotypic differences reflect the adaptations that the bacteria undergo in CF airways (Folkesson A et al, 2012). The major adaptations of *P.aeruginosa* in CF airways are discussed in this section and summarized in table 4.

Mucoid phenotype. The most studied adaptation of *P. aeruginosa* in CF is the mucoid phenotype (biofilm mode of growth) (Høiby N, 2011). The mucoid phenotype is due to overproduction of the exopolysaccharide alginate, a polymer of D-manuronic acid and L-guluronic acid (Govan JRW and Deretic V et al, 1996). Most of the alginate biosynthetic genes are located in a single operon, the

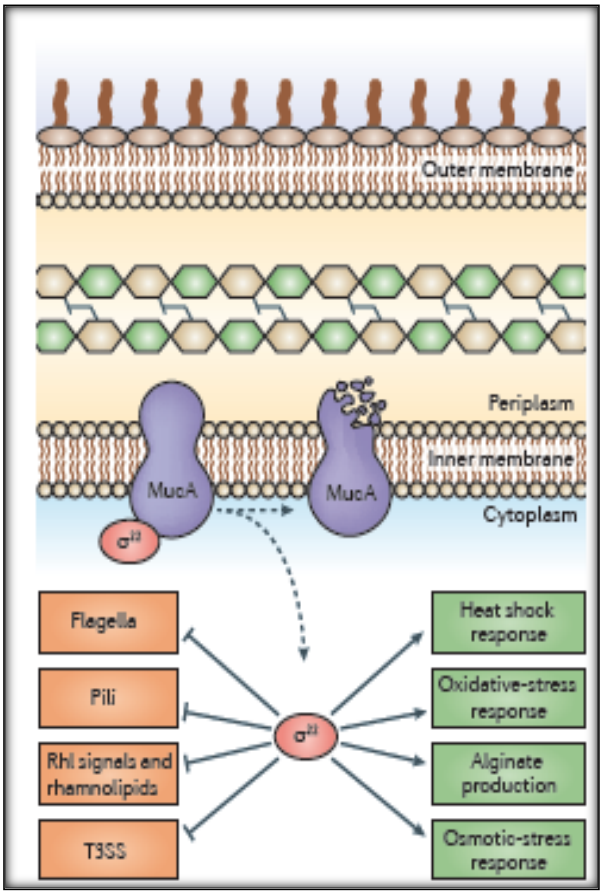


Figure 14. *Regulation network of MucA–σ22. The function of the RNA polymerase σ-factor σ22 (encoded by algU) is antagonized through protein–protein binding by the anti-σ-factor MucA. Mucoid Pseudomonas aeruginosa isolates from CF patients carry knockout mutations in mucA, leaving σ22 free to activate transcription of many genes, such as those involved in alginate production and the responses to heat shock, osmotic stress and oxidative stress. σ22 also negatively regulates several virulence factors, including flagella, pili, the type III secretion system (T3SS) and Rhl quorum sensing signals, as well as rhamnolipids that are controlled by Rhl.*

algD cluster (May TB and Chakrabarty AM. 1994), that is subjected to both positive and negative regulation. The *algD* promoter requires the sigma factor AlgT (also called AlgU) for expression (Schurr Mj et al, 1996). AlgT is bound and sequestered by the anti-sigma factor MucA, which itself is encoded by the *mucA* gene. Thus, MucA normally limits the expression of the *algD* gene cluster and the production of alginate. However, after extended periods in the airways of patients with CF, *P. aeruginosa* acquires mutations in the *mucA* gene, which results in loss of MucA and high levels of AlgT. This leads to unbridled expression of the *algD* cluster and overproduction of alginate and the mucoid phenotype. Overproduction of alginate favours the establishment of *P. aeruginosa* chronic infection in the context of CF in several ways (Hauser AR et al, 2011). It favors the biofilm mode of growth thus preventing bacterial clearance by both host phagocytes and antibiotic therapy (Hentzer M et al, 2001).

Alternatively, by forming a capsule around *P. aeruginosa* cells, alginate may impede opsonization, phagocytosis and thus killing (Song Z et al, 2003). Moreover, alginate may contribute to dysregulate the host immune response (Hauser AR et al, 2011). The mucoid phenotype is relatively common in respiratory samples from CF patients. It emerges following an average of 3 years of infection (Mahenthiralingam E et al, 1994) and at a median age of 13 years (Li Z et al, 2005), but can be observed as soon as 3 months after infection (Govan, JR and Nelson JW, 1992.) and as early as 18 months of age (Li Z et al, 2005). Interestingly, 70% of nonmucoid isolates from chronically infected CF patients carry mutations in the *mucA* gene, suggesting that these strains at one time were mucoid but had reverted to a nonmucoid phenotype (Ciofu O et al, 2008). Often chronic infection with mucoid *P. aeruginosa* is associated with poor prognosis in CF patients. However, it is unclear at the moment whether the mucoid phenotype actually causes poor clinical outcomes or rather is a marker for highly adapted strains that have increased virulence due to other mutations. Another possibility is that the mucoid phenotype is simply selected because of the hypoxic lung environment found in advanced CF disease (Hauser AR et al, 2011).

Table 4. *Adaptations of P. aeruginosa observed during chronic respiratory infections of CF patients.*

Adaptation	Mutated gene(s)
Mucoid colony morphology	*mucA*
Antibiotic resistance	*mexZ*, *mexA*, *mexT*, *ampD*
Lipopolysaccharide alterations	*pagL*, O-antigen biosynthetic cluster (*himD*/*ihfB* to *wbpM*)
Loss of type III secretion	*exsA*, *vfr*, *cyaB*
Loss of motility	*rpoN*, *vfr*, *fleQ*, *pilB*, *pilQ*, *cyaB*
Auxotrophy and metabolic changes	*lasR*
Small-colony variants	*wspF*
Defective quorum sensing	*lasR*
Hypermutability	*mutS*, *mutL*, *uvrD*
Loss of pyoverdin secretion or uptake	Deletion of pyoverdin synthetic and uptake genes
Loss of pyocin production	
Changes in pyocyanin production	
Peptidoglycan modification	
Loss of type II secretion or altered activity of secreted factors (e.g., exotoxin A, phospholipase C, elastase)	*toxA*, *toxR*

(Hauser AR et al, 2011)

Antibiotic resistance. Antimicrobial resistance is common in *P. aeruginosa,* like in most CF pathogens. Slow bacterial growth and reduced metabolic activity, in addition to the physical barrier of the biofilm matrix, all contribute to this phenomenon. Moreover, CF pathogens frequently undergo mutations in genes controlling the production of efflux pumps and β-

lactamases (Smith EE et al, 2006) thus creating antibiotic-resistant lineages of *P. aeruginosa* that expand under the selective pressure of antimicrobial therapy.

P. aeruginosa actively eliminates antibiotics by enzymatic cleavage as in the case of β-lactam antibiotics or by efflux mechanisms. Four multidrug efflux systems have been reported to play a role in the antibiotic resistance of *P. aeruginosa* CF isolates, the MexXY-OprM system playing the predominant role in aminoglycoside resistance (Callaghan M and McClean S, 2012). Thus, although high doses of antibiotics are delivered to the lung of CF patients, P. *aeruginosa* is difficult to eliminate with antibiotic therapy (Pitt TL et al, 2003).

Modification of LPS. LPS plays an important role during *P. aeruginosa* infection due to its strategically location at the interface of pathogen with the pulmonary environment. This structure is often modified in *P. aeruginosa* isolates from CF patients, suggesting a role in the establishment of chronic infection (figure 15). Loss of O-antigen, which may facilitates the chronic persistence within the respiratory tract, has been detected. It results from the accumulation of different mutations, such as small frameshift deletions or integration of insertion elements, in the cluster of biosynthetic genes responsible for O-antigen production or by deletion of all or large parts of this locus (Evans DJ et al,1994; Ernst RK et al, 2003; Kresse AU et al, 2003; Spencer DH et al, 2003). More recently, it has been shown that the LPS lipid A portion is also altered in CF isolates. *P. aeruginosa* isolates from chronically infected CF patients synthesize lipid A with distinctive acylation patterns. Whereas lipid A from environmental or non-CF *P. aeruginosa* isolates is predominantly penta-acylated (i.e., contains five acyl groups embedded within the lipid bilayer of the outer membrane), CF isolates contain substantial amounts of hexa- and hepta-acylated (containing six or seven acyl groups) lipid A (Ernst RK et al, 1999; Ernst RK et al, 2003; Cigana C et al, 2009). Moreover, the lipid A from *P. aeruginosa* CF isolates also contains residues of aminoarabinose, a positively charged amino sugar (Ernst RK et al, 2007).

Lipid A modifications may have important biological consequences. For instance, addition of aminoarabinose enhances resistance to antimicrobial peptides and to some antibiotics (Ernst RK et al, 1999). Acylation affects LPS recognition and signalling through human Toll-like receptor 4 (TLR4) and the subsequent inflammatory response (Alexander C and Rietschel ET, 2001; Hajjar AM et al, 2002). Indeed, it has been reported that LPS from late *P. aeruginosa* CF isolates induces a reduced inflammatory response than LPS from an early isolates from the same patient (Cigana C et al, 2009).

Loss of type III secretion. The majority of *P. aeruginosa* isolates from the environment and from acute infections have the ability to inject into the cells a set of toxic effector proteins that includes ExoS, ExoT, ExoU, and ExoY through a type III secretion system (Hauser AR et al, 2011). This system is activated on direct contact with eukaryotic cell membrane and interferes with signal transduction, resulting in cell death or alterations in host immune response (Sadikot RT et al, 2005).

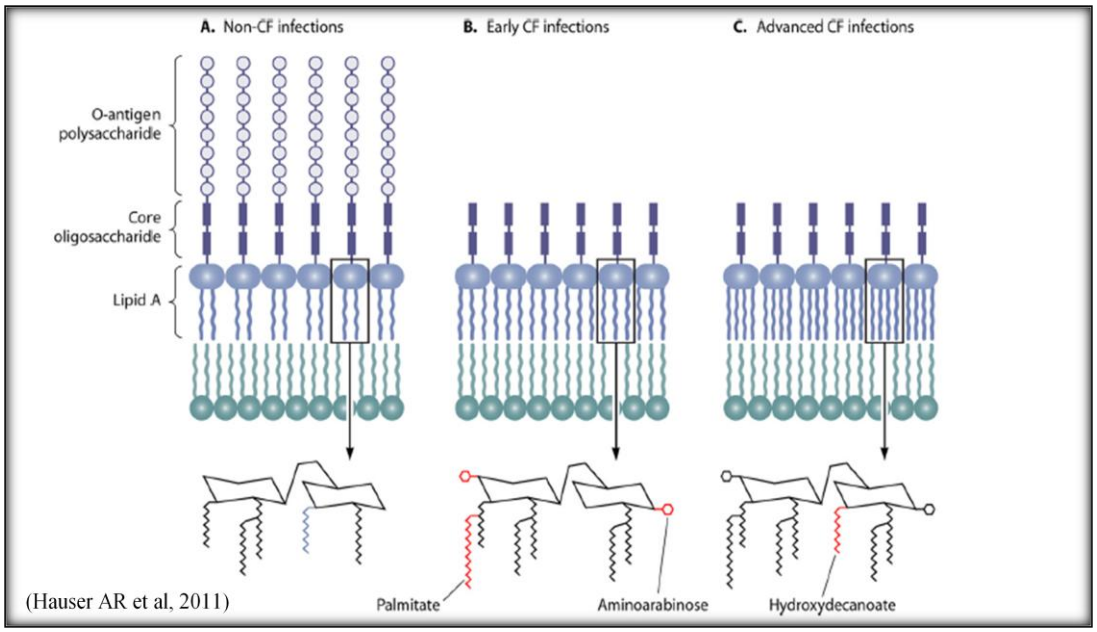


Figure 15. *Modifications of P. aeruginosa LPS during CF respiratory infections. (A) Structures of LPSs from strains recovered from the environment, acute infections, or bronchiectasis. The lipid A, core oligosaccharide, and O-antigen polysaccharide components are indicated. In the lower panel, a more detailed structure of lipid A is shown. The large polygons represent the diglucosamine bisphosphate backbone of lipid A, and the staggered lines represent acyl groups. (B) During early infection in individuals with CF, the O-antigen polysaccharide is frequently lost. Also, palmitate (shown as a red staggered line) and aminoarabinose (shown as a red hexagon) are added to the lipid A portion of LPS. (C) In patients with advanced CF, a hydroxydecanoate chain (shown as a red staggered line) is retained, likely due to mutations in the pagL gene, which encodes a lipid A deacylase*.

During infection of the CF lungs, however, *P. aeruginosa* strains gradually lose the ability to secrete these effector proteins (Dacheux D et al, 2000; Jain M et al, 2004; Lee V et al, 2005). Several factors promote the persistence of *P. aeruginosa* clones that lost type III secreted proteins. Individuals with CF mount an antibody response against type III proteins (Moss J et

al, 2001; Banwart B et al, 2002; Corech R et al, 2005). Therefore, over time secretion-positive strains are cleared from CF patients whereas secretion-negative strains are not. Moreover, the effects that type III toxins have on the host, may not be consistent with long-term bacteria persistance within the human respiratory tract. This idea is supported by the paucity of CF isolates that harbour the *exoU* gene, which encodes the most cytotoxic of the type III secreted proteins (Feltman H et al, 2001; Jain M et al, 2004; Lanotte P et al, 2004).

Loss of motility. *P. aeruginosa* strains cultured from the airways of patients with CF often are defective in motility. This loss of motility occurs during the course of infection, since *P. aeruginosa* isolates cultured early during infection are motile. *P. aeruginosa* CF isolates are reported to be defective both in swimming motility and twitching motility. Particularly, *P. aeruginosa* strains from CF airways often fail to produce fully functional flagella, thus making the bacteria defective in swimming motility (Mahenthiralingam E et al, 2004). The basis for this gradual loss of swimming motility appears to be related to acquisition of mutations in one of several genes that regulate flagella production, including *rpoN*, *vfr*, and *fleQ* (Smith EE et al, 2006), although downregulation of the *fliC* gene (which encodes flagellin, the structural subunit of the flagellum) in response to CF airway fluid may also play a role (Wolfgang Mc et al, 2004; Jyot J et al, 2007). TLR5 recognizes flagellin and signals for upregulation of the proinflammatory response (Cobb LM et al, 2004; Hybiske K et al, 2004). Accordingly loss of flagella, and related swimming motility, decreases immune recognition of the pathogen by TLR5 (Zhang, Z et al, 2005) favouring resistance to phagocytosis (Mahenthiralingam E et al, 2004) and contributing to the establishment of chronic infection.

Many strains of *P. aeruginosa* are capable of movement over surfaces by twitching motility. This process is mediated by the extension and retraction of type IV pili, proteinaceous filamentous appendages on the surface of *P. aeruginosa* (Mattick JS, 2002). Chronic infection in CF is associated with loss of twitching motility by several mechanisms. Mutations in the *pilB* gene disrupt production of the PilB protein, which is essential for pilus biogenesis (Kresse AU et al, 2003; Smith EE et al, 2006). Likewise, mutation of the *pilQ* gene, which encodes a transmembrane protein essential for extrusion of the pilus through the bacterial outer membrane, may occur (Chang YS et al, 2007). Finally, strains from chronically infected patients may lack RpoN, a sigma factor necessary for production of type IV pili (Mahenthiralingam E et al, 1994; Smith EE et al, 2006).

Auxotrophy and metabolic adaptations. *P. aeruginosa* respiratory isolates from CF patients frequently grow slowly on defined laboratory media, suggesting the presence of defective metabolic pathways (Head NE and Yu H, 2004). Indeed it has been reported that a high percentage of *P. aeruginosa* CF isolates are auxotrophs (Taylor RF et al, 1992; Taylor RF et al, 1993). Moreover, auxotrophs and prototrophs of the same genotype are often detectable in CF airways indicating that the auxotrophs probably derive from the wild-type bacteria (Barth AL and Pitt TL**,** 1995). The high concentration of free amino acids in CF respiratory secretions apparently allows these auxotrophic strains to survive during infection and obviate the need for biosynthetic pathways (Barth AL and Pitt TL, 1996; Son MS et al, 2006). Consistent with this notion is the increased expression of genes involved in arginine uptake and its metabolism in some CF isolates (Hoboth C et al, 2009). Other metabolic adaptations can occur in the CF environment. Indeed *P. aeruginosa* is able to metabolize various carbon and nitrogen sources and can grow in either aerobic or anaerobic environment using several terminal electron acceptors. The presence of cyanide in CF sputum suggests that, at least in some districts of the CF lung, *P. aeruginosa* grows under microaerophilic conditions. Moreover, *P.aeruginosa* isolates have increased transcription of genes involved in both denitrification and fermentation. Thus it appear that *P. aeruginosa* well adapts to anaerobic niches that can be found in the CF lungs (Ryall B et al, 2008).

Small-colony variants (SCV). *P. aeruginosa* can form SCVs in the context of CF. Because they take more than 48 h to appear on culture plates, SCVs are easily missed in clinical practice, but they are thought to be present in about 10% of the respiratory specimens of CF patients (Schneider M et al, 2008). *P. aeruginosa* SCVs have special characteristics: they auto aggregate in liquid culture, are hyperadherent to surfaces, exhibit reduced motility, and importantly, often have enhanced resistance to antibiotics (Boles BR et al, 2004; Deziel E et al, 2001; Drenkard E and Ausubel FM, 2002; Haussler S et al, 2003). These features promote a biofilm mode of growth *in vitro* and are thought to do the same in the CF airways (Deziel E et al, 2001; Kirisits MJ et al, 2005). Mutations or changes in expression of chemosensory (*wspF*), exopolysaccharide (*psl* and *pel*), and two-component system (*pvrR*) response regulators may contribute to the SCV phenotype in *P. aeruginosa* (D'Argenio DA et al, 2002; Drenkard E and Ausubel FM, 2002; Kirisits M et al, 2006; Starkey M et al, 2009), although current evidence suggests that individual SCVs differ significantly from one another in their

gene expression patterns. Thus, SCVs may represent a heterogeneous group of bacteria that share only a subset of their phenotypes (Haussler S et al, 2003; Kirisits MJ et al, 2005).

Defects in quorum sensing. The most extensively studied *P.aeruginosa* quorum sensing systems are LasI/LasR system and RhlI/RhlR system producing the autoinducers 3-oxo-dodecanoyl homoserine lactone and butyryl homoserine lactone, respectively (Parsek MR and Greenberg EP, 2000). These systems play an important role in the pathogenesis of *P. aeruginosa* infection of the CF lung through the regulation of virulence factors and promotion of biofilm formation. Indeed it has been reported that *in vitro* quorum-sensing systems modulate expression of 6 to 10% of the genes in the *P. aeruginosa* genome (Worlitzsch D et al, 2002; Schuster M et al, 2003), including virulence determinants such as elastase, alkaline protease, phospholipase C, pyocyanin, and exotoxin A (Storey, DG et al, 1998; Schaber JA et al, 2004; Girard G and Bloemberg GV, 2008; .Hoboth C et al, 2009). However, it has been found that many *P. aeruginosa* isolates from CF patients fail to produce homoserine lactones (Lee B et al, 2005; Smith EE et al, 2006; D'Argenio DA et al, 2007; Wilder CN et al, 2009). Accordingly, 3-oxo-dodecanoyl homoserine lactone is not detectable in the sputa from 22 to 46% of CF patients infected with *P. aeruginosa* (Chambers CE et al, 2005; Erickson DL et al, 2002; Middleton B et al, 2002). It is now clear whether mutations in the *lasR* and *rhlR* genes account for loss of quorum sensing in many CF isolates. These mutations usually occur about 15 years following the onset of lung infection (Bjarnsholt T et al, 2010). Several explanations have been proposed for these apparently paradoxical findings. The metabolic cost of producing the large number of factors under the control of quorum sensing systems is high, thus some clones of bacteria do not themselves respond to autoinducers but benefit from the autoinducer factors synthesized by their neighbours (Diggle SP et al, 2007; Sandoz KM et al, 2007). It may simply be that quorum sensing is not required (or may even be detrimental) once chronic infection is established. Alternatively mutations in *lasR* may confer an advantage in CF by altering the gene expression patterns and leading to an increased growth rate and increased resistance to antibiotics. For example, *lasR* mutants produce higher levels of β-lactamases compared to wild types bacteria, resulting in increased tolerance to β-lactam antibiotics (D'Argenio DA et al, 2007; Hoffman LR et al, 2010). Moreover quorum sensing-dependent production of virulence determinants may accounts for loss of virulence systems such as type III secretion and flagella are lost once chronic infection ensues. In any case, it

appears that the role of *P. aeruginosa* quorum sensing in CF is more complex than initially anticipated.

Hypermutable phenotype

Hypermutable strains are those isolates that have mutation rates higher by 20 to 1,000 fold respect to reference strains. Hypermutable isolates have been identified among CF pathogens, including *P. aeruginosa, H. influenzae* and *S. aureus* (Callaghan M and McClean S, 2012). *P. aeruginosa* hypermutable strains result from mutations in the *mutS*, *mutL*, and *uvrD* genes, which encode proofreading proteins responsible for correcting errors that occur during DNA replication (Miller JH 1996; Mena A et al, 2008; Wiegand I et al, 2008). Hypermutable strains become more common in the later stages of infection (Oliver A et al, 2000; Hogardt M et al, 2007; Ciofu O et al, 2010). They are more resistant to antibiotics (Oliver A et al, 2004; Macia MD et al, 2006; Wiegand I et al, 2008; Ferroni, A et al, 2009), more likely to be mucoid (Moyano AJ et al. 2007; Waine DJ et al, 2008) or defective in quorum sensing (Lujan AM et al, 2007), more metabolically adapted to the CF airways (Hoboth, C et al, 2009), and in general more adaptable to the harsh environment of the CF airways (Hauser AR et al, 2011). Moreover the presence of hypermutator strains in the bacterial populations of the CF airways is often associated with the parallel occurrence of different subpopulations with different phenotypic traits (Folkesson A et al, 2012).

In summary, *P. aeruginosa* apparently does not require many of its factors to persist in the CF lungs. Indeed, many of the adaptations involve the gradual loss of virulence factors crucial for acute infections. Thus, it is not surprising that *P. aeruginosa* isolates from chronically infected CF patients are less virulent than clonal isolates collected from the same patients at earlier stages of infection and even than other *P. aeruginosa* strains when tested in animal models of acute infection (Burke V et al, 1991; Bragonzi A et al, 2009). This attenuation of virulence probably helps the bacteria to survive to the host immune response by eliminating factors detected by the host and by causing less tissue damage, which itself may stimulate an inflammatory response. In addition, strains not burdened with the production of multiple virulence determinants may be able to grow more rapidly and thus have a fitness advantage (Oberhard MA et al, 2010). Finally, many of the adaptive phenotypes are interrelated. For example, MucA regulates not only the mucoid phenotype but also type III secretion (Wu w et al, 2004; Jones AK et al, 2010) and indirectly flagellum genes (Garrett ES et al, 1999; Tart

AH et al, 2005; Tart AH et al, 2006). LPS modifications (Ernst RK et al, 1999; Ernst RK et al, 2006), the SCV phenotype (Haussler S, 2004), and defects in quorum sensing (D'Argenio DA et al, 2007; Hoffman LR et al, 2010; Robinson TE et al, 2009) are all associated with increased resistance to antibiotics. A proportion of SCVs exhibit auxotrophy (Starkey M et al, 2009), and quorum sensing indirectly regulates type III secretion (Hogardt M et al, 2004). Thus, a single mutation may lead to a number of adaptive phenotypes. Although adapted strains of *P. aeruginosa* have enhanced survival in CF it remains unclear whether these adapted strains in turn cause more severe clinical disease

Aim

Patients with cystic fibrosis are characterized by chronic lung disease due to persistent bacterial infection and inflammation (Davis PB, 2001). For a long time the cause of hypersusceptibility of CF lung to bacterial infections has been attributed to dysfunctions of the airway epithelial cells which express high level of the CFTR on their apical membranes and actively contribute to the defence in the lung. More recently an increasing body of data indicates that other cells of the immune system, such as neutrophils and macrophages, are affected by CFTR mutations suggesting their contribution in CF lung pathology. Indeed, in the murine model it has been recently demonstrated that macrophages carrying mutated CFTR display defective bactericidal activity and exaggerated secretion of pro-inflammatory cytokines.

In order to assess the contribution of CFTR in the functional activity of human macrophages the principal aim of my PhD thesis has been to verify the expression and the functional activity of CFTR in human macrophages and to determine the contribution of this protein in the bactericidal activity of phagocytes. First, I have demonstrated that human macrophages express CFTR that, as in epithelial cells, functions as a chloride channel. Next, the influence of dysfunctional CFTR in the bactericidal activity of such phagocytes has been addressed by comparing the intracellular bacterial survival in CF and non CF macrophages infected by *P. aeruginosa*. By this approach, I demonstrated that, similarly to murine macrophages, human CF macrophages displayed a reduced capability to eliminate intracellular *P. aeruginosa* compared to non CF cells.

At the present is unknown which microbicidal mechanisms are responsible for *P. aeruginosa* killing by macrophages. Since it has been reported that CFTR dysfunction in murine alveolar macrophages compromises NADPH oxidase assembly and activation, thus reducing reactive oxygen species mediated *P. aeruginosa* killing, I have subsequently investigated the oxidative burst response and its role in bacterial killing by human control and CF macrophages.

Additionally, I studied the role of the cathepsins and matrix metalloproteinases (MMPs) in the killing of the engulfed bacteria. Cathepsins are very important lysosomal enzymes involved in the degradation of engulfed pathogens by phagocytes, whereas MMPs are supposed to function extracellularly or on the cell surface. However, MMPs have recently been implicated in intracellular processes including apoptosis, cell cycle regulation and intracellular antimicrobial activity (Houghton AM et al, 2009). The contribution of the proteases to

intracellular bacteria killing was analyzed by using different protease inhibitors to treat macrophages before infection with *P.aeruginosa.* Then, survival of bacteria within untreated and treated cells was determined.

The work reported here was performed using different cellular models of human origin including monocyte derived macrophages (MDM), lung macrophages and macrophagic cell lines.

Materials and Methods

Study subjects

Blood was obtained from 14 healthy donors and from 12 patients with Cystic Fibrosis confirmed by positive sweat tests and detection of CF-inducing mutations (5 males, 7 females, median age 31).

All CF-patients were clinically stable at the time of blood donation and did not receive systemic antibiotic or corticosteroids treatment. Nine CF patients were F508del homozygous, one was W1282X homozygous, and two carried at least one delta F508del allele (F508del/D192G and F508del/P5L).

Lung macrophages were isolated from 17 people with cystic fibrosis (9 males, 8 females, median age 30) and 13 control individuals (6 males, 7 females, median age 53) attending the Department of Thoracic Surgery, Sapienza University, Policlinico Umberto I, Rome, Italy. Eight CF patients were F508del homozygous, seven were F508del heterozygous, one was N1303K/K119R and one was W1282X homozygous. Samples of lung parenchyma were obtained from subjects with cystic fibrosis undergoing a double lung transplant; lung tissue resections were obtained from non-CF patients undergoing thoracic surgery. All patients gave written informed consent and the study was approved by the local ethics committee (Comitato Etico,Azienda Policlinico Umberto I, Rome, Italy; 21 June 2007).

Isolation and differentiation of human monocytes

Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Lympholyte, Cedarlane, Hornby, CA). $CD14^+$ cells were purified from PBMC by positive selection with anti-CD14 mAb coupled to magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of $CD14^+$ was routinely 90% as estimated by flow cytometry using FITC conjugated CD14 (BD Biosciences). $CD14^+$ cells were differentiated for 7 days in RPMI 1640 (Gibco-BRL, Invitrogren Corporation Carlsbad,CA, USA) supplemented with 20% FCS and 100 ng/ml recombinant macrophage colony stimulating factor (MCSF; PeproTech Inch, Rocky Hill, NY, USA) at 37 °C in a humidified 5% CO 2 atmosphere.

Isolation of human lung macrophages

Lung parenchyma was abundantly washed with PBS 0.1% EDTA and then minced finely with scissors. Dispersed cells were collected and filtered over sterile gauzes. Cells were centrifuged at 500g for 10 minutes and macrophage suspension was enriched by flotation over Percoll (GE) density gradients. A 90% (v/v) solution of Percoll was generated by mixing 9

parts of Percoll with 1 part of PBS 10X (Sigma). Gradients of 60, 50, 40, 30 and 20% were prepared by diluting the 90% (v/v) solution of Percoll with PBS. The cell pellet was resuspended in 5 ml of the 50% Percoll solution and carefully layered onto 60% Percoll solution in a 50 ml tube. A six-step discontinuous gradient was prepared by overlaying the 50% Percoll solution with cells followed by 5 ml of the 40, 30, 20% Percoll solutions. On top of the gradient, 2.5 ml of PBS were layered and the gradient was centrifuged at 21 °C, for 25 min and at 630 *g* without brake. Recovered cells were suspended at $0.5x10^6$ cells/ml in RPMI 1640 (Gibco-BRL, Invitrogren Corporation Carlsbad, CA, USA) supplemented with 5% FCS and incubated in 48-24 multi-well plates (Falcon BD Biosciences) at 37°C in a humidified atmosphere at 37°C and 5% CO_2. After overnight incubation, adherent cells were detached and staining with anti-CD68 was assessed by flow cytometry.

To assess the morphology of adherent cells, cytospin preparations were made on glass slides by centrifugation at 500*g* for 2 minutes and stained with Diff-Quick. The images were acquired with Nikon Eclipse E400, using 20x objective.

Intracellular staining for CD68

Cells, $3x10^4$, were washed in PBS and then resuspended in 100 µl of cytofix/cytoperm solution (BD, Bioscience) for 20 min at 4°C. Cells were washed and the anti-CD68-FITC antibody (AbD Serotech) was added for 30 m at 4°C. Cells incubated with the IgG1-FITC isotype served as controls. Cells were then washed, resuspended in PBS before flow cytometry analysis (FACS Calibur) using CellQuest software (Becton Dickinson).

Cell culture

THP-1 cells, ATCC TIB-202**,** were cultured in RPMI 1640 (Euroclone) supplemented with 10% FCS (Euroclone), 2 mmol/L L-glutamine (Sigma) and 100 units/mL penicillin/streptomycin (Sigma) at 37 °C in a humidified 5% CO_2 atmosphere. THP-1 cells were seeded at $4x10^5$ cells/ml and differentiated into macrophages using 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 3 days. After the initial 3 days, PMA containing media was removed and the cells were incubated in fresh medium for a further 4 days.

Cells were collected, washed with PBS and incubated with the anti-CD14-FITC antibody (BD Pharmigen) and anti-CD11b-phycoerytrin antibody (BD Pharmigen) for 30 min. Cells incubated with the IgG1-FITC isotype (RD system) and IgG1- phycoerytrin isotype (RD system) served as controls. Analyses were performed using the FACS Calibur (BD Bioscience) and data were analyzed with the CellQuest software (BD Bioscience).

RNA extraction and real time PCR

Total RNA was isolated from macrophages by Tryzol (Tryzol Reagent, Invitrogren Corporation Carlsbad, CA, USA), treated with DNase and purified using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. cDNAs were obtained using Reverse Trascription System kit (Promega, Fitchburg, WI, USA) and random primers. RNA quality and concentration were determined by electrophoresis and spectrophotometry. Real time PCR were done using SYBR Green PCR Master Mix (Applied Biosystem, Forster City, CA, USA) according to the suppliers' specification. The primers were as follow: for CFTR, rtCF-F1 5'-AAGCGTCATCAAAGCATGCC-3'(cDNA nt position 1686/1705) and rtCF-R1 5'-TTGCTCGTTGACCTCCACTCA- 3' (cDNA nt position1775/1795); for the actin gene (used as the endogenous reference gene) rtbeta-ActF1 5'- GCCGGGACCTGACTGACT-3' and rtbeta-ActR1 5'- TGGTGATGACCTGGCCGT-3'. Each sample was amplified in triplicates and CFTR mRNA level was determined by the ΔCt relative quantification method (7300 System SDS software, Applied Biosystem).

Bactericidal assay

The day before infection, macrophages were seeded in 48 well plates ($1x10^5$ cells/well). *P. aeruginosa*, strain ATCC 27853, was grown over night in nutrient broth medium, harvested by centrifugation, washed twice in PBS and resuspended in RPMI 1640 (Gibco-BRL, Invitrogren Corporation Carlsbad, CA, USA) supplemented with 5% FBS. Macrophages were infected at a multiplicity of infection (MOI) of 20-30, i.e. 20-30 bacteria for one macrophage. Bacteria were brought in contact with macrophages by centrifugation (500 g for 10 min). The end of centrifugation was considered the starting point of infection which proceeded for 1 hr at 37°C in 5% CO_2. After infection, the cells were gently washed with PBS (three times) and incubated for 1 hr in culture medium containing gentamycin (400 μg/ml) to kill the extracellular bacteria. The end of this step was codified as t0. Some wells were lysed to determine the number of intracellular bacteria at t0; others were incubated in antibiotic-free medium for additional four hours; samples were taken after 2 (t2) and 4 (t4) hours from t0. At each time point the cells were lysed with 1% Triton X-100 (Sigma) in PBS and the bacteria were counted by plating serial dilutions of the lysates on nutrient agar plates.

When indicated, macrophages were pre-treated with DPI (10μM) or L-NAME (100μM) for 30 min before infection, infected with Pa27853 for 1 hr and treated with antibiotics for 30

min to kill the extracellular bacteria. CFU recovered from control or treated cells were normalized to the input CFU.

The day before infection, THP-1 differentiated cells were seeded in 96 well plates ($1x10^5$ cells/well). *P. aeruginosa*, strain PaO1, was grown over night in nutrient broth medium, harvested by centrifugation, washed twice in PBS and resuspended in RPMI 1640 (Euroclone) supplemented with 5% FBS (Euroclone). Cells were pre-treated with 50μM E64 (Sigma), 100μM PepstatinA (Milllipore), 150μM Pefabloc (Sigma), 1mM EDTA (Sigma), 20μM GM6001(Milllipore) and 20μM Phosphoramidon (Sigma) for 1 hr before infection, infected with PaO1 for 1 hr at MOI of 10 and treated with culture medium containing streptomycin (200 μg/ml) for 1hr to kill the extracellular bacteria. The cells were treated again with 50μM E64, 100μM PepstatinA, 150μM Pefabloc, 1mM EDTA, 20μM GM6001 and 20μM Phosphoramidon during antibiotic treatment. Following antibiotic treatment, intracellular bacteria were enumerated as described before.

Measurement of reactive oxygen species (ROS)

Luminol chemioluminescence assay was used to detect intracellular and extracellular ROS generated by monocyte-derived macrophages (MDM) upon interaction with *P. aeruginosa*. MDM ($1,5x10^5$ cells) resuspended in HBSS (Hanks' balanced salt solution) were seeded on 96-well microtitre plates and pre-opsonized (5% FCS) bacteria were added to the wells (MOI, 15) in the presence of 250 μM of Luminol (3-Aminophthalhydrazide, 5-Amino-2,3-dihydro-1,4- phthalazinedione, Sigma). The microtitre plates were centrifuged at 100 g for 2 min at 21°C to synchronyze the infection. The end of centrifugation was considered the start of reaction. The relative amount of ROS generated by macrophages was detected at regular interval over 80 min by measuring the luminescence with Perkin Elmer Victor 2 Microplate Reader. The flavoprotein inhibitor diphenyleneiododium (DPI, Sigma) was added to selected wells at the final concentration of 5 μM. Intracellular ROS was detected by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA; Invitrogen). Macrophages were incubated with CMH2DCFDA, 4 μM, in HBSS for 30 min at 37°C. After incubation cells were washed and infected with *P. aeruginosa ATCC 27853* at a MOI of 20 for 1h at 37°C. Postinfection, cells were centrifuged for 5 min at 500g, resuspended in PBS and stained with propidium iodide (Sigm) before flow cytometer analysis (FACS Calibur) using CellQuest software (Becton Dickinson).

THP-1 differentiated cells pre-treated for 1hr with GM6001/Phosphoramidon and un-treated cells were incubated with 1 μM CMH2DCFDA in PBS for 5 min at 37°C. After incubation cells were washed and infected with PAO1 at MOI of 30 or treated with 10 μM N-formyl-Methionyl-L-leucyl-L-phenylalanine (fMPL, Sigma) for 20 minuts at 37°C. DPI, (Sigma) was added when indicated at the final concentration of 10 μM. Postinfection, cells were centrifuged for 5 min at 500g, resuspended in PBS and stained with propidium iodide (Sigma) before flow cytometer analysis (FACS Calibur) using CellQuest software (Becton Dickinson).

Determination of lysosomal pH

P. aeruginosa, PaO1 strains, was grown over night in nutrient broth medium, harvested by centrifugation and washed twice in PBS. Bacteria were resuspended in PBS at the concentration of $1x10^9$ CFU/ml, treated with 10μg/ml BCECF-AM (Sigma) and incubated at 30 °C rocking for 1 hours. Bacteria were harvested by centrifugation, washed twice in PBS and resuspended in PBS at the concentration of $1X10^9$ CFU/ml. THP-1 cells differentiated into macrophages were detached from plate and resuspended in RPMI 1640 (Euroclone) supplemented with 5% of FBS (Euroclone). THP-1 cells were pre-treated with 20μM of phosphoramidon and 20μM of GM6001 for 1 hours, infected at MOI of 30 and incubated for 1 hr at 37 °C in 5% CO_2. After infection, the cells were gently harvested by centrifugation and incubated for 1 hr in culture medium containing streptomycin (200 μg/ml) to kill the extracellular bacteria. The cells were gently harvested by centrifugation, resuspended in PBS and transferred in 96 MW plates.

BCECF-AM was successively excited at 490 nm and 440 nm, and the resultant fluorescent signal was monitored at 535 nm using Perkin Elmer Victor 2 Microplate Reader.

Fluorescence intensity ratio (490/450) data were converted into pH values using the bacterial calibration curve done by incubation of bacteria treated with BCECF-AM in standard pH buffer (pH range 5 to 8,5). The fluorescence intensity ratio data thus obtained were converted into pH values using the equation pH=pKa-log (R-Ra)/(Rb-R)*Fa(l2)/Fb(l2), where R is the ratio F(λ1)/F(λ2) of fluorescence intensities (F) measured at two wavelengths λ1=490nm and λ2 =450 nm and the subscripts A and B represent the limiting values at the acidic and basic endpoints, respectively, previously generated from calibration experiments.

Determination of cytosolic pH

THP-1 differentiated cells were detached, harvested by centrifugation and washed in PBS. Cells were resuspended in PBS at the concentration of $1,5x10^6$ /ml, treated with 5μg/ml

BCECF-AM (Sigma) and incubated at room temperature rocking for 30 minuts. The cells were gently harvested by centrifugation, resuspended in PBS and transferred in 96 MW plates. Some cells were treated with nigericine (Sigma) to generate the calibration curve and the pH values were determined as described before for lysosomal pH.

Statistical analysis

Statistical analysis was performed by GraphPad Prism 4 software (GraphPad software Inc.) and the statistical tests are shown in the figure legends. Cases and controls P values less than 0.05 were considered statistically significant.

Results

PART I: Human monocyte derived macrophages express CFTR which contributes to their bactericidal activity against *P. aeruginosa*

With the aim to verify whether CFTR contributes to the antimicrobial activity of human phagocytes first we have verified the expression and the functional activity of CFTR in human macrophages. By real-time PCR, immunofluorescence and whole cell patch clamp we have demonstrated that CFTR localizes at the plasma membrane of human non CF macrophages where it acts as a cAMP dependent channel. Next the contribution of CFTR in the bactericidal activity of human macrophages was investigated by performing antibiotic protection assays over a four hour period in CF and non-CF macrophages. Results from this analysis allowed to demonstrate that, although a progressive reduction of live intracellular bacteria was observed both in CF and non-CF cells, a significantly higher number of surviving bacteria was rescued from CF cells four hours after infection supporting the notion that CFTR plays a role in the eradication of bacteria by macrophages. The results of this part of the work are detailed in annex 1.

Annex 1

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PLoS one

Dysfunctional CFTR Alters the Bactericidal Activity of Human Macrophages against *Pseudomonas aeruginosa*

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Abstract

Chronic inflammation of the lung, as a consequence of persistent bacterial infections by several opportunistic pathogens represents the main cause of mortality and morbidity in cystic fibrosis (CF) patients. Mechanisms leading to increased susceptibility to bacterial infections in CF are not completely known, although the involvement of cystic fibrosis transmembrane conductance regulator (CFTR) in microbicidal functions of macrophages is emerging. Tissue macrophages differentiate *in situ* from infiltrating monocytes, additionally, mature macrophages from different tissues, although having a number of common activities, exhibit variation in some molecular and cellular functions. In order to highlight possible intrinsic macrophage defects due to CFTR dysfunction, we have focused our attention on *in vitro* differentiated macrophages from human peripheral blood monocytes. Here we report on the contribution of CFTR in the bactericidal activity against *Pseudomonas aeruginosa* of monocyte derived human macrophages. At first, by real time PCR, immunofluorescence and patch clamp recordings we demonstrated that CFTR is expressed and is mainly localized to surface plasma membranes of human monocyte derived macrophages (MDM) where it acts as a cAMP-dependent chloride channel. Next, we evaluated the bactericidal activity of *P. aeruginosa* infected macrophages from healthy donors and CF patients by antibiotic protection assays. Our results demonstrate that control and CF macrophages do not differ in the phagocytic activity when infected with *P. aeruginosa*. Rather, although a reduction of intracellular live bacteria was detected in both non-CF and CF cells, the percentage of surviving bacteria was significantly higher in CF cells. These findings further support the role of CFTR in the fundamental functions of innate immune cells including eradication of bacterial infections by macrophages.

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Introduction

Cystic fibrosis is the most common genetic disorder affecting the Caucasian population. The disease is caused by mutations of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) which encodes a c-AMP dependent chloride channel [1,2]. Defective chloride secretion due to dysfunctions of CFTR results in the dehydratation of airway liquid leading to depletion of the periciliary layer and production of highly viscoelastic mucus which significantly impact mucociliary clearance and expectoration [3]. This combination of factors prevents the elimination of bacteria from the lung permitting bacterial infections to become established. Bacterial infections eventually lead to chronic inflammation, which accounts for the progressive tissue damage and ultimately to respiratory failure in CF [4]. Indeed CF lung inflammation is characterized by a sustained accumulation of neutrophils, high proteolitic activity and elevated levels of pro-inflammatory cytokines and chemokines [5]. Besides alteration of the mucociliary clearance system, CFTR mutations might affect other functions of bronchial epithelial cells including the internalization of *P. aeruginosa*, the release of inflammatory mediators, sphingolipid metabolism and transport of the redox buffer molecule GSH [6–9].

Recently, several reports suggest that CFTR might be of pivotal importance in the normal function of immune cells, such macrophages and neutrophils. For instance, Painter at al. have shown that CFTR expressed on phagolysosomes is crucial for the chlorination reactions involved in bacterial killing by human neutrophils [10,11]. Studies in the CF knockout mice demonstrated that CFTR participates in phagosomal pH control of murine alveolar macrophages, thereby CFTR-deficient macrophages failed to acidify lysosomes and phagolysosomal compartments and displayed an altered bactericidal activity [12,13]. Additionally, defects in the ROS mediated killing of *P. aeruginosa* by murine CFTR-deficient alveolar macrophages have been recently reported by Zhang et al [14]. This defect has been associated with the

failure of infected macrophages to activate acid sphimgomyelinase and to release ceramide, thus preventing the formation of ceramide enriched membrane platforms that serve to cluster and activate NADPH oxidase [14].

Finally, a contribution of CFTR in the production of different cytokines by macrophages has been recently described. Bruscia et al. showed that in response to *P. aeruginosa* lipopolysaccharide (LPS), bronchoalveolar fluids from CF mice present significantly higher concentrations of macrophage derived pro-inflammatory cytokines such as IL-1α, IL-6, G-CSF and IL-8 as compared to wild type cells. Results from *in vitro* stimulation of alveolar and bone marrow derived macrophages with *P. aeruginosa* LPS confirmed the exuberant cytokine production in CF cells [15].

Although these data support the hypothesis that the abnormal macrophage activity, due to the lack of CFTR might be one of the causes of persistent bacterial infections and exuberant inflammatory responses in CF, at present the contribution of CFTR in the physiology of human macrophages is unknown. In order to define possible intrinsic macrophage defects due to CFTR deficiencies we have analyzed the bactericidal activity against *P. aeruginosa* of human monocyte derived macrophages from CF patients. To this aim we have first verified CFTR expression and functional activity in macrophages from healthy donors, subsequently, we have evaluated the capacity of CF macrophages to kill intracellular *P. aeruginosa*. Our results show for the first time that, macrophages derived from peripheral blood monocytes isolated from healthy donors express a functional CFTR and efficiently kill intracellular bacteria. Comparison of the bactericidal activity of control and CF macrophages revealed a significant increase in bacterial survival in cell carrying dysfunctional CFTR suggesting that CFTR, independently from tissue origin, directly contributes to microbicidal function of phagocytes.

Materials and Methods

Study subjects

Fifteen patients with CF (Regional Cystic Fibrosis Center, Sapienza University, Rome Italy), confirmed by positive sweat tests and genotyping (6 males, 9 females, median age 27) were enrolled in the study. Clinical and demographic characteristics of CF patients are reported in Table 1. Blood samples, for isolation of $CD14^+$ cells, were collected when patients attended the clinic for routine evaluation. Informed written consent was obtained from all participants after approval of the study by the local ethics committee (Comitato Etico, Azienda Policlinico Umberto I, Rome, Italy; 21 June 2007). Blood samples from twelve, sex and age matched healthy donors were used as controls in bacterial infection assays. Additionally twelve healthy donor samples were used in RT-PCR experiments.

Isolation and differentiation of human monocytes

Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Lympholyte, Cedarlane, Hornby, CA). $CD14^+$ cells were purified from PBMC by positive selection with anti-CD14 mAb coupled to magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of $CD14^+$ was routinely >90% as estimated by flow cytometry using FITC conjugated CD14 (BD Biosciences). $CD14^+$ cells were differentiated for 7 days in RPMI 1640 (Gibco-BRL, Invitrogren Corporation Carlsbad, CA, USA) supplemented with 20% FCS and 100 ng/ml recombinant macrophage colony stimulating factor (MCSF; PeproTech Inch, Rocky Hill, NY, USA).

RNA extraction and real time PCR

Total RNA was isolated from macrophages by Tryzol (Tryzol Reagent, Invitrogren Corporation Carlsbad, CA, USA) treated with DNase and purified using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. cDNAs were obtained using Reverse Trascription System kit (Promega, Fitchburg, WI, USA) and random primers. Real time PCRs were done using SYBR Green PCR Master Mix (Applied Biosystem, Forster City, CA, USA) according to the suppliers' specification. The primers were as follow: for CFTR, rtCF-F1 5′-AAGCGTCATCAAAGCATGCC-3′(cDNA nt position 1686/1705) and RTCF-R1 (5′-TTGCTCGTTGACCTCCACTCA-3′ (cDNA nt position1775/1795); for the actin gene (used as the endogenous reference gene) rtbeta-ActF1 5′-GCCGGGACCTGACTGACT-3′ and rtbeta-ActR1 5′-TGGTGATGACCTGGCCGT-3′. Each sample was amplified in triplicates and CFTR mRNA level was determined by the ΔΔCt relative quantification method (7300 System SDS software, Applied Biosystem). Either parental monocytes or the alveolar epithelial cell line H441 (ATCC, HTB-174), which expresses low level of CFTR mRNA, were used as calibrators.

Table 1. Characteristics of CF patients.

Patient	Age	Sex	Genotype	Microbiology[a]	FEV1%
CF1	25	M	F508del/-	none	98%
CF2	34	F	F508del/F508del	*S.a.*	76%
CF3	16	F	F508del/F508del	none	87%
CF4	20	F	F508del/F508del	*S.a.*	49%
CF5	24	F	F508del/R1162X	*S.a,P.a.*	63%
CF6	34	F	F508del/L732X	*C.a, S.a.,P.a.*	33%
CF7	15	M	F508del/F508del	*A.t., S.m.*	39%
CF8	22	M	F508del/N1303K	*S.a., P.a.*	39%
CF9	23	M	F508del/F508del	*P.a.*	91%
CF10	33	F	F508del/F508del	*P.a.,P.f.*	49%
CF11	27	F	F508del/F508del	*P.a.*	28%
CF12	45	F	F508del/F508del	*P.a.*	44%
CF13	30	M	W1282X/W1282X	*P.a.*	43%
CF14	28	M	F508del/F508del	*S.a.*	66%
CF15	36	F	F508del/F508del	*S.a.*	76%

[a]*S.a.: Staphylococcus aureus; P.a.: Pseudomonas aeruginosa; C.a.: Candida albicans; A.t.: Aspergillus terreus; S.m.: Stenotrophomonas maltophilia; P.f.: Pseudomonas fluorescens;* - unknown;
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Immunofluorescence and Confocal Laser Scanning Microscopy (CLSM) analyses

For CLSM analyses, 1×10^5 *in vitro* differentiated macrophages were seeded in 24-well cluster plates on cover glasses (diameter, 12 mm) the day before analysis. Cells were fixed with paraformaldeyde 3% (PFA, 30 min 4°C) permeabilized with 0.5% Triton X-100 (10 min, room temperature) and then stained at 37°C with the anti-CFTR polyclonal antibody H-182 (Santa Cruz Biotechnology, Santa Cruz California), followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG F(ab)2 (Molecular Probes, Eugene, OR). The cover glasses were extensively washed with PBS 1× and mounted on the microscope slide with Vectashield antifade mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). To determine CFTR localization in the lysosomes, macrophages were first stained for CFTR using as secondary antibody Alexa Fluor-594 goat anti-rabbit IgG F(ab)2, and then they were labelled with the mouse anti human LAMP-1

antibody (Developmental Studies Hybridoma Bank, University of IOWA, IOWA City, IA) and the Alexa Fluor-488 F(ab)2 goat anti-mouse IgG. CLSM observations were performed with a Leica TCS SP2AOBS apparatus using excitation spectral laser lines 405, 488 and 594 nm and using a 63× oil immersion lens. Image acquisition and processing were achieved using the Leica Confocal Software (LCS) (Leica Lasertechnik, Heidelberg, Germany) and Adobe Photoshop software programs (Adobe system, Mountain View, CA). Signals from different fluorescent probes were taken in sequential scan settings, and co-localization was detected in yellow (pseudo-colour). At least 50 individual cells were analyzed for each staining condition. Isotype control antibodies were used in all confocal microscopy experiments to confirm the specificity of antibody staining.

Electrophysiology recordings

All experiments were conducted at room temperature (22–24°C) using an Axopatch 200B patch clamp amplifier (Axon Instruments, Burlingame, CA, USA) and using the pCLAMP 9.0 and CLAMPFIT 9.0 as acquisition and data analyses programs respectively (Axon Instruments, Burlingame, CA, USA). Patch-clamp pipettes, obtained using borosilicate glass (Science Products GmbH, Hofheim, Germany), and a Heka puller (model PIP5, HEKA Elektronik, Lambrecht/Pfalz Germany), had resistances between 8–10 MOhms. Whole-cell recordings were performed according to the method previously described by Hamill et al. [16]. *In vitro* differentiated macrophage cells were plated on 35 mm Petri dishes at density of 1×10^5 cells/dish, and, during the current recordings, bathed utilizing solutions in which Cl^- was the only possible permeable ion according to the protocol previously described for epithelial cells [17]. The pipette solution contained (in mM): 140 N-methyl D-glucamine; 40 HCl; 100 L-glutamic acid; 0.2 $CaCl_2$; 2 $MgCl_2$; 1 EGTA; 10 HEPES; and 2 ATP-Mg, pH 7.2. The bath solution contained (in mM): 140 N-methyl D-glucamine; 140 HCl; 2 $CaCl_2$; 1 $MgCl_2$; and 10 HEPES, pH 7.4. Cells were stimulated by the addition of a cAMP-activating cocktail (400 μM cAMP, 10 μM forskolin, 1 mM IBMX) to the pipette solution with voltage steps ranging from −110 to +110 mV for 200 ms with increments of 10 mV from a holding potential of −40 mV.

Bactericidal assay

This assay was performed as described by Auriche C. et al. with minor modifications [18]. Briefly, the day before infection, macrophages were seeded in 48 well plates (1×10^5 cells/well). *P. aeruginosa*, strain ATCC 27853, was grown over night in tryptic soy medium, harvested by centrifugation, washed twice in PBS 1× and once in serum-free RPMI 1640 medium before being re-suspended in RPMI 1640 supplemented with 10% FBS at $\sim10^7$ CFU/ml. Based on preliminary experiments and previously published data macrophages were infected at a multiplicity of infection (MOI) of 30, i.e. 30 bacteria for one macrophage [12,18]. Bacteria were brought in contact with macrophages by centrifugation (500 g for 10 min). The end of centrifugation was considered the starting point of infection which proceeded for 1 hr at 37°C in 5% CO_2. After infection, the cells were gently washed with PBS 1× (three times) and incubated for 1 hr in culture medium containing gentamycin (400 mg/ml each) to kill the extracellular bacteria. The end of this step was codified as t0. Some wells were lysed to determine the number of intracellular bacteria at t0; others were incubated in antibiotic-free medium for additional four hours; samples were taken after 2 (t2) and 4 (t4) hours from t0.

Intracellular bacteria were counted by lysing the cells with 1% Triton X-100 in PBS 1× and plating serial dilutions of the lysates on PIA plates (Pseudomonas Isolation Agar). The fraction of internalized bacteria was determined with respect to the CFU used to infect the cells (the input), whereas bacterial survival was determined with respect to the CFU recovered at t0.

Each experimental section included the following controls: wells of uninfected macrophages; wells inoculated with bacteria only. Data were considered only when the following conditions were confirmed: lysates from un-infected macrophages did not revealed any bacterial colony on PIA plates; bacteria alone incubated for 1 hr with 400 mg/ml gentamycin were completely killed (<10 CFU left). Viability of macrophages after infection was determined by trypan blue counting in representative experiments and Annexin V staining.

Statistical analysis

Mann-Whitney nonparametric test was used to investigate the significance of differences on bacteria counts between cases and controls at the different time-points analyzed. The same test was performed to evaluate differences on bacteria survival between cases and controls (at t2 and t4). *P* values less than 0.05 were considered statistically significant.

All the statistical procedures were performed by STATA 11 statistical package.

Results

CFTR expression in macrophages and precursor cells

As first, we wanted to assess whether the *CFTR* was expressed by *in vitro* differentiated macrophages and parental monocytes. For this purpose total RNA was extracted from both cell populations obtained by twelve healthy donors and CFTR mRNA was detected by real time PCR. The resulting data were analyzed by the relative quantification method using as calibrators either the low expressing control cells H441 or the parental monocytes (Fig. 1A and 1B). Both monocytes and macrophages showed CFTR expression, however the level of the CFTR transcript was higher in macrophages with respect to parental monocytes. Indeed, in most of the macrophage populations analyzed (8/12) we detected 2–15 fold increase of the CFTR mRNA in macrophages with respect to parental monocytes, in one sample we didn't observe significant variation and in 3 samples we observed a reduction down to half of that observed in monocytes (Fig. 1B). Overall these data demonstrate that *in vitro* differentiated macrophages express CFTR.

Expression and functional activity of CFTR protein in differentiated macrophages

Having demonstrated the presence of CFTR mRNA in monocyte derived macrophages, we analyzed the expression of CFTR protein by immunofluorescence using the anti-CFTR antibody H-182 (Santa Cruz), raised against the first 182 aa of the protein. Examination of *in vitro* differentiated macrophages from control individuals by confocal microscopy showed that the majority of cells expressed CFTR which mainly localized at the plasma membranes or in their vicinity (Fig. 2A and 2B). Additionally, detectable intracellular staining was observed in a small percentage of the cells (Fig. 2A). By contrast, in CF macrophages homozygous for F508del mutation, the global immunofluorescence signal was much lower with respect to wild type (wt) cells and the brightest CFTR staining was found in the cytoplasm indicating a predominant intracellular localization of the protein (Fig. 2A).

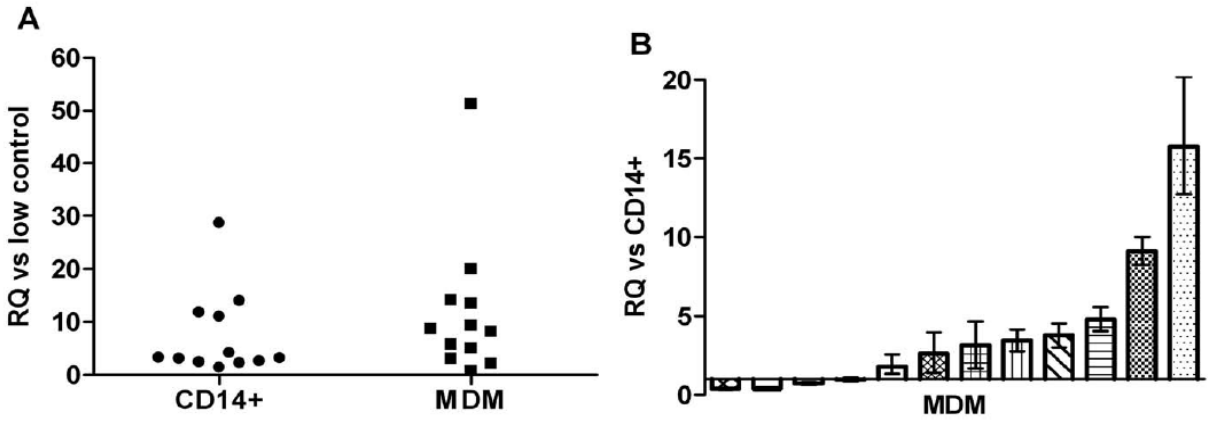

Figure 1. CFTR mRNA expression in human *in vitro* differentiated macrophages from non-CF donors. Panel A: Mean relative quantitiy (RQ) of CFTR mRNA in monocytes ($CD14^+$) and monocyte derived macrophages (MDM) calibrated *versus* the H441 cells (low control); each symbol represents a single donor. Panel B: CFTR mRNA in MDM (mean RQ) calibrated *versus* parental monocytes. Each bar represents a single donor, whiskers above and below are the RQ-max and RQ-min, respectively.
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In order to verify whether CFTR was also localized in some lysosomal vescicles of MDM, we double stained control MDM for CFTR and for the lysosomal-associated membrane protein 1 (LAMP1). Results from this analysis confirmed the plasma membrane localization of CFTR but they did not allow to demonstrate any co-localization of CFTR with the lysosomal marker as previously reported in murine lung macrophages (Fig. 2B).

To test if the antibody revealed protein was also functional, whole cell patch-clamp analyses were performed utilizing solutions in which chloride was the only mobile ion. This experimental protocol allowed to record currents from the entire CFTR channel population expressed on the plasma membrane of macrophages (basal; panel A Fig. 3). The presence of cAMP-containing cocktail, after 2–4 min of the establishment of the whole cell patch clamp configuration, stimulated a twofold increase in Cl^- currents with respect to the basal condition (cAMP, panel A Fig. 3). The treatment with the thiazolidinone $CFTR_{inh\text{-}172}$, a selective blocker of the cystic fibrosis transmembrane conductance regulator, reverted the cAMP-evoked Cl^- currents (cAMP/$CFTR_{inh\text{-}172}$, panel A Fig. 3). The graph in panel B of figure 3 shows the current/voltage relationship indicating that the currents appeared voltage independent with a chloride-selective reversal potential. The current density observed under basal conditions was $3{,}54 \pm 0{,}45$ pA/pF, this value increased up to $6{,}67 \pm 0{,}88$ pA/pF in cAMP-induced activation conditions, while in presence of $CFTR_{inh\text{-}172}$ the obtained current density was $3{,}15 \pm 0{,}96$ pA/pF, a value similar to that observed in basal conditions (panel C, Fig. 3).

Bactericidal activity in wt and CF macrophages

Our data clearly demonstrate that CFTR is expressed and it is functionally active as a Cl^- channel in monocyte-derived macrophages isolated from healthy donors. Next, we determined the bactericidal activity of monocyte-derived macrophages from healthy donors (non-CF) and CF individuals against *P. aeruginosa*. A descriptive panel of the patients tested for macrophage bactericidal activity is reported in Table 1, in addition healthy donors matched for sex and age were included as controls. Overall the bactericidal activity was assayed in 15 CF samples and 12 non-CF controls.

The bactericidal activity of macrophages was assayed using the antibiotic protection method over a 4 hr time period. After infection, intracellular live bacteria were detected by the colony-forming unit (CFU) method at three time points: at the end of infection; two and four hours after infection. In order to ensure identical experimental conditions at least one non-CF and one CF sample were evaluated in each experimental section. The first data we have analysed was the number of intracellular live bacteria at the end of infection, which could be influenced by the phagocytic capacity of macrophages. The median number of live bacteria recovered from infected macrophages were 2108 and 1506 in HD and CF macrophages respectively with no significant differences between the two groups ($P = 0{,}9611$) suggesting that the phagocytic activity of these cells was very similar.

Next we determined the percentage of live bacteria two and four hours after infection with respect to the bacteria recovered at the end of infection. Two hours after infection, non-CF macrophages caused a rapid decline in the fraction of intracellular live bacteria down to a median percentage of survival of 26,4. Similarly, but to a lesser extent, CF macrophages reduced the percentage of intracellular live bacteria down to 33,34 (Fig. 4). Statistical evaluation of these results failed to reveal significant differences between the two groups at this time point. Only two macrophage samples were found to diverge from the majority: one non-CF sample showed an increase in the number of live bacteria two hours after infection of about two order of magnitude with respect to the other HD samples; similarly, at the same time point post-infection, one CF showed an increased number of live bacteria but by a factor of 10 (Fig. 4 and Fig. 5). However, both samples showed a subsequent reduction of intracellular live bacteria. Of note these samples have been analyzed in different experimental sections together with samples that behaved as the majority of macrophages. In the next time point analysed (four hours after infection) live bacteria recovered from non-CF macrophages were significantly less than those recovered from CF macrophages. Indeed the median percentage of live bacteria recovered from HD and CF macrophages was 16,90 and 25 respectively; ($P = 0{,}0359$). Although the deficit in the killing activity of CF versus non-CF macrophages was not as severe as previously reported for murine alveolar macrophages [12,13] this result

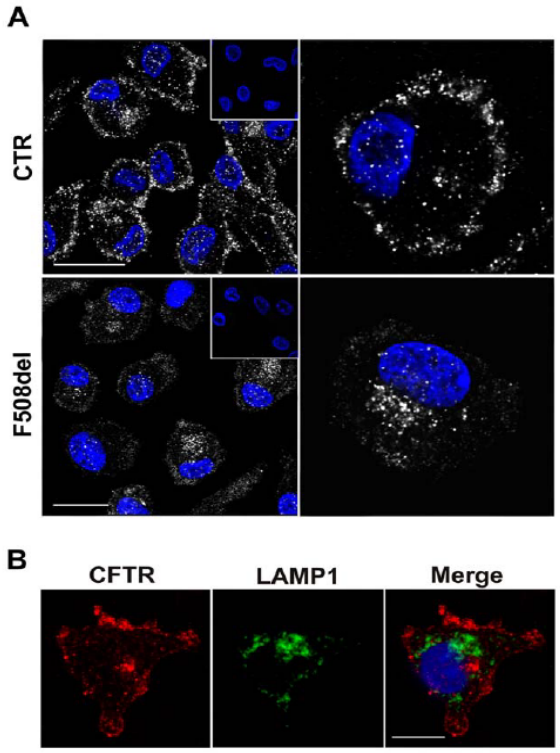

Figure 2. Confocal microscopy of the cellular localization of CFTR in control and F508del human *in vitro* differentiated macrophages. (A) Localization of CFTR in macrophages from non-CF (top row) and del508F homozygous CF individuals (bottom row). Permeabilized macrophages were stained with the polyclonal anti-CFTR antibody (H-182) and with Alexa Fluor 488-conjugated secondary antibody. Nuclei were counterstained with DAPI. Scale bars = 20 micron. Isotype negative controls are shown in the insets. (B) Permeabilized macrophages were stained with the anti-CFTR and the anti-LAMP1 antibodies; the secondary antibodies were Alexa Fluor-594 and Alexa Fluor-488 conjugated F(ab)2 IgG. Scale bar = 10 micron.
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strongly suggests that CF macrophages do indeed display intrinsic deficiency of bactericidal activity. Furthermore, counting of viable macrophages and evaluation of apoptosis by annexinV staining (data not shown) failed to reveal differences in cell viability during the time course of the experiments excluding that the reduction in surviving bacteria was due to cell death. It has to be pointed out that the cellular model we have used for this analysis consists of macrophages which were not conditioned by the lung environment and thereby represent the best system to highlight possible intrinsic deficiencies of CF macrophages.

Discussion

Chronic inflammation of the lung, as a consequence of persistent bacterial infections by several opportunistic pathogens represents the main cause of mortality and morbidity in CF patients [19]. At present the causes of the inability of CF patients to eradicate bacterial infections have been mainly ascribed to dysfunctions in the defence mechanisms mediated by airway epithelial cells. More recently, several studies in the murine model demonstrated that dysfunctional CFTR might alter the bactericidal activity of alveolar macrophages, further contributing to the poor control of bacterial growth in CF patients [12–14,20]. In order to evaluate whether a similar defect affects human macrophages, we have compared the capacity of monocyte derived macrophages (MDM) from CF and control individuals to kill intracellular *P. aeruginosa*. The choice of this model was dictated by our primary goal i.e. analysis of possible intrinsic defects of macrophages which requires macrophagic cells unconditioned by the lung environment.

Due to the lack of available data, first we verified the expression and activity of CFTR in human macrophages by different techniques; i.e. RT-PCR, immunofluorescence, patch clamp recording. Our immunofluorescence localization data on MDM from healthy individuals demonstrated that wild type CFTR is predominantly localized at the plasma membranes or in the vicinity of them in the majority of MDM, with a small percentage of cells showing an intracellular localization. In contrast the same analysis on F508del homozygous cells revealed a strong reduction of plasma membrane staining compared to that observed in the cytoplasm. This latter finding might be explained by the elimination of misfolded CFTR by cellular quality control mechanisms. Indeed deletion of the F508 residue, which represents the most prevalent mutation in the CFTR, causes a temperature sensitive folding defect leading to protein degradation by the endoplasmic reticulum associated degradation machinery [21]. In addition our data are in agreement with previous reports showing that F508del CFTR protein mislocalizes from the apical membranes to the cytoplasm in primary airway and nasal epithelial cells [22,23].

Consistently with plasma membrane localization of CFTR, whole-cell patch-clamp recordings from MDM from healthy individuals showed that the presence of Cl^- currents increased in the presence of an intracellular cAMP stimulation. As expected, the currents recorded were sensitive to $CFTR_{inh\text{-}172}$, which has been reported to specifically block CFTR Cl^- conductance, supporting the results that the CFTR channels, not only are localized at plasma membrane, but are also functional [24]. Peak current density measured from human MDM during cAMP activation revealed lower values than those recorded by Di and collaborators from human alveolar macrophages [12]. This difference could be due to a different functional status of the analysed cell population; in addition, also different levels of CFTR on plasma membrane, due to different tissue origins, could be postulated.

It has been previously reported that CFTR is present not only at the plasma membranes but also within phagosomes, lysosomes and possibly other intracellular compartments in murine lung macrophages [12,14]. Our results from double staining of permeabilized MDM with anti-CFTR and the anti-lysosomal marker LAMP1 did not support the expression of CFTR in lysosomes of human MDM. Differences between our data and those reported by Zhang and collaborators showing that CFTR localizes with some LAMP1 positive vesicles in freshly isolated lung macrophages might be explained either by differences in the cellular localization of CFTR in distinct species or in cells from different anatomical sites (periphery *vs* lung), or both. However, we cannot exclude that CFTR localization in lysosomal vesicles couldn't be detected by immunofluorescence due to low sensitivity of the method, indeed in the J774 murine macrophage cell line, immunoelectron microscopy was employed to co-localize CFTR and LAMP1 [12].

The role of CFTR in regulating bactericidal activity by macrophages was first demonstrated in 2006 in murine macrophages, this defect was observed in lung, but not peritoneal, macrophages and was related to the inability of CFTR−/− macrophages to maintain an acidic pH in the intralysosomal

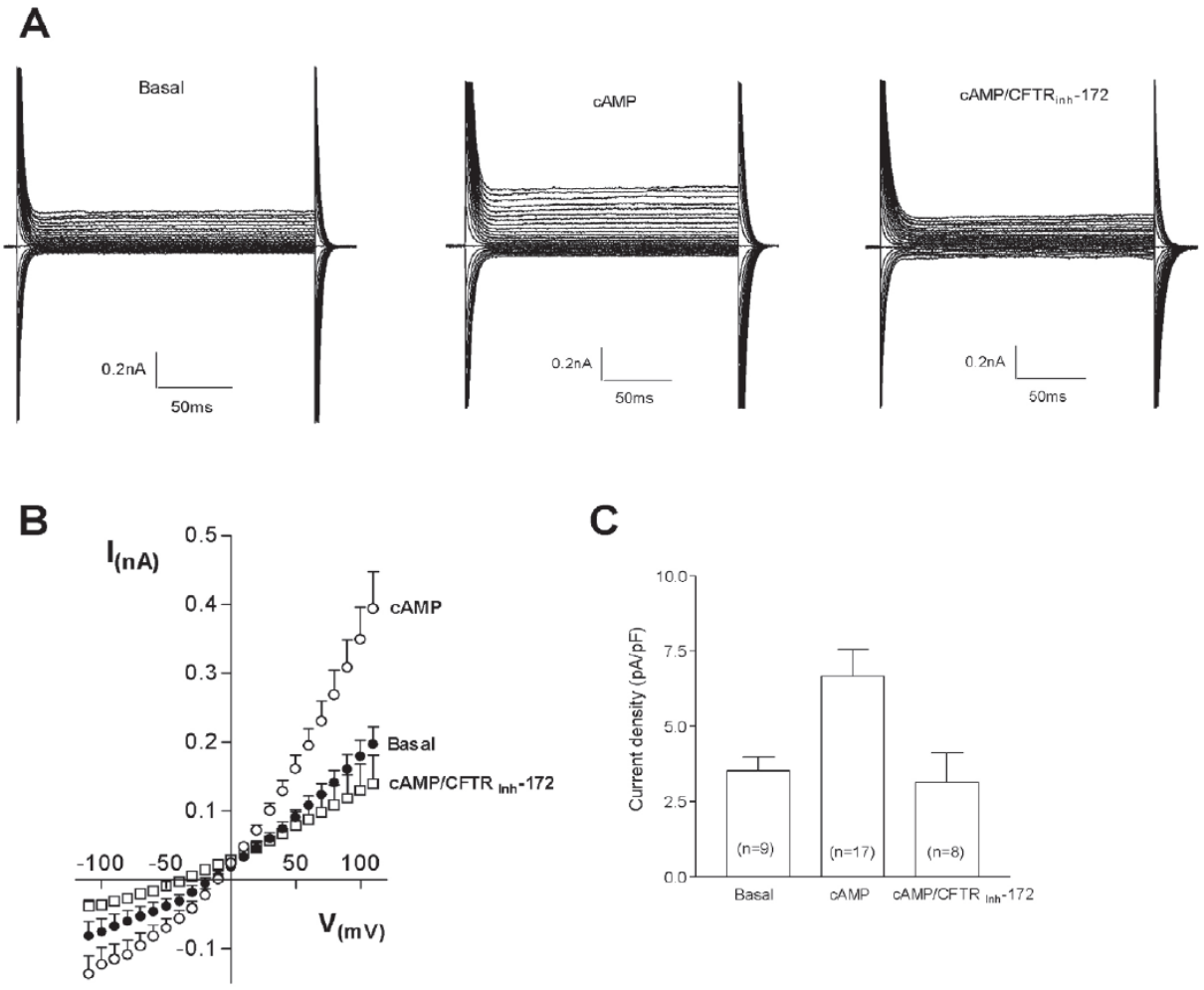

Figure 3. Whole-cell patch clamp of cAMP-evoked Cl^- current in human peripheral macrophages. (A) representative currents in basal condition (basal) or in response to the administration the cAMP-containing cocktail (cAMP), recorded on voltage-clamped macrophages. cAMP-evoked currents were blocked in the presence of the specific inhibitor $CFTR_{inh-172}$ (10 μM), added to the bath solution (cAMP/$CFTR_{inh-172}$). Currents were recorded in 200 ms voltage steps from −110 to +110 mV with 10 mV increments from a holding potential of −40 mV. (B) Averaged current/voltage relationship in basal conditions (basal, n = 9), in the presence of a cAMP-containing cocktail (cAMP, n = 16) or cAMP-containing cocktail plus 10 μM $CFTR_{inh-172}$ (cAMP/$CFTR_{inh-172}$, n = 7). (C) Current densities obtained at +110 mV in the three described experimental conditions, in parentheses the number of recorded cells are shown. Data reported in panel (B) and (C) are means±S.E.
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compartments [12]. Subsequent studies, by others as well as by the same group, produced contrasting results and the involvement of CFTR in lysosomal acidification is still debated [13,25–28]. More recently measurements of ROS release, following *P. aeruginosa* infection, by WT and CFTR-deficient murine alveolar macrophages revealed a deficiency in ROS production and *P. aeruginosa* killing in CF macrophages. It has to be noted that Di and collaborators failed to reveal differences in ROS production by alveolar macrophages isolated from WT and CFTR-deficient mice [12]. Although the different experimental conditions might be responsible for these contradictions, whether CFTR activity has a direct role in phagosomal acidification and the mechanism/s responsible for macrophage dysfunction, are still opened questions. In this scenario, and due to the very few data on human macrophages, we have focused our attention on non-conditioned phagocytic cells, such as MDM, and studied the ability to kill *P. aeruginosa*. First, we determined whether differences could be found in the fraction of internalized bacteria at the end of infection between WT and CF macrophages. Statistical analysis of our data failed to reveal any significant difference suggesting that the phagocytic activity of monocyte derived macrophages is not affected by CFTR. Next, we determined the outcome of the intracellular bacteria over a period of 4 hour after infection. In the first 2 hours after infection, the fraction of live bacteria decreased at similar extend in WT and CF macrophages. On the contrary 4 hours after infection, although live bacteria continued to decrease, those recovered from CF macrophages were significantly more than from control cells. Thereby, for the first time to the best

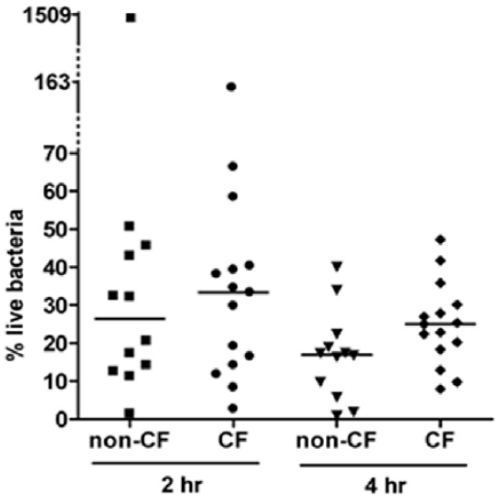

Figure 4. Surviving bacteria within human macrophages. Percentage of intracellular live bacteria rescued from *P. aeruginosa* infected macrophages two (2 hr) and four (4 hr) hours after infection. 100% refers to bacteria recovered at the end of infection (t0). Samples: non-CF, healthy donor macrophages (N = 12); CF, macrophages from cystic fibrosis patients (N = 15). Each symbol represents a single individual, the line is the median percentage of live bacteria.
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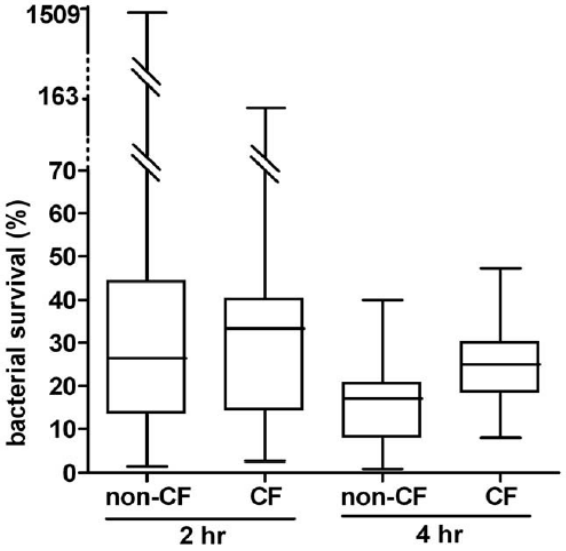

Figure 5. Summary data of live intracellular bacteria. The percentage of surviving bacteria two (t2) and four (t4) hours after infection with respect to live bacteria recovered at the end of infection (t0). Data are expressed as box plots representing, the 25 and 75 percentiles, median, minimal and maximal values. Statistical analysis: non-CF *vs* CF, $P = 0{,}7697$ and $P = 0{,}0359$ two and four hours after infection, respectively.
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of our knowledge, we show that the bactericidal activity of human macrophages is indeed affected by CFTR-deficiency. Defective clearance of apoptotic cells has been previously reported in human MDM when exposed to the aqueous sol fraction of sputum recovered from CF patients. This impairment has been associated with the cleavage of phosphatidylserine receptor in a neutrophil elastase dependent manner [29]. Our data highlights a novel dysfunction of human CF MDM which, since it cannot be ascribed to conditioning by CF lung environment, reflects a primary defect of CF MDM. It is interesting to note that the observed defect in the bactericidal activity of human CF MDM was less profound than that previously demonstrated in murine CF alveolar macrophages. A possible explanation for this finding is the existence of multiple bactericidal mechanisms employed by human MDM to kill *P. aeruginosa* whose, only a part might be affected by CFTR deficiency as previously reported for human neutrophils [11]. Two strong clinical correlations have been identified in CF: i) a positive correlation between persistent *P. aeruginosa* infection and worst prognosis for the patients ii) a marked adaptation of the bacteria to the CF lung by a number of changes including loss of bacteria motility, altered antibiotic susceptibilities and metabolic shift (decreased oxygen consumption and increased nitrate utilization) [30–33]. Since our data were obtained with a *P. aeruginosa* strain, that might not recapitulate the phenotypes of chronic isolates it is reasonable to surmise that the observed deficit of bactericidal activity of CF macrophages could be more pronounced when challenged with late clinical isolates. It is generally accepted that phagocytic cells use a combination of oxidative and non-oxidative mechanisms to defend against a great variety of engulfed microorganisms [34]. Having demonstrated that the microbicidal activity of CF macrophages is significantly reduced four hours after infection but not at the previous time point (two hours), it might be hypothesised that multiple bactericidal mechanisms operating with different kinetics are differently affected by CFTR activity. Experiments are underway to analyse the molecular pathways leading to bacteria killing in WT and CF MDM.

Author Contributions

Conceived and designed the experiments: MAM PDP FA. Performed the experiments: NC SG FS SG MA EGDD. Analyzed the data: NC SG FS SG MA MAM PDP FA. Contributed reagents/materials/analysis tools: MAM FS FA PDP SQ. Wrote the paper: PDP FA. Identified and provided clinical samples: FV SQ.

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PART II: The bactericidal activity of lung macrophages from CF patients is impaired

Isolation and characterization of lung macrophages

To further address the role of CFTR in human macrophages activity, I extended the analysis of the bactericidal activity to CF lung macrophages. To this aim I set up a method to isolate macrophages from lung parenchyma obtained from CF patients undergoing lung transplantation. Control non-CF cells were isolated from chirurgic specimens of non-CF patients undergoing thoracic surgery. Confirmation that the isolated cells corresponded to lung macrophages was obtained by the analysis of cell morphology and expression of the monocyte/macrophage marker CD68 by flow cytometry. Results from immunofluorescence demonstrated that more than 90% of the isolated cells were positive to the anti-CD68 antibody (figure 16 A). Additionally, Quick-Dif staining revealed that the selected cells had the characteristic morphology of macrophages (figure 16 B).

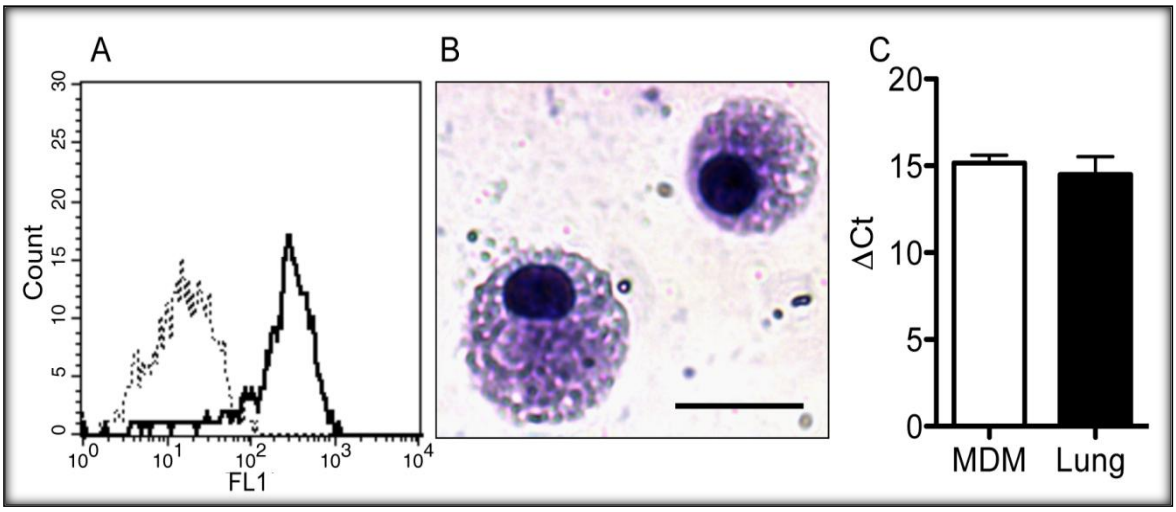

Figure 16. *Human lung macrophages express cftr.*

A)FACS analysis of macrophages stained with anti-CD68. Dashed Line, unstained sample.

B)Lung macrophages isolated from parenchyma stained with DIF-stain. Scale bar is equal to 20 μm.

C)CFTR mRNA expression in macrophages isolated from lung parenchima (lung, N=4) or from monocyte derived macrophages (MDM, N=8) as detected by real time PCR.

Moreover, CFTR expression in control non-CF lung macrophages was determined by real time PCR. Results obtained from macrophages isolated from 4 non-CF subjects showed an average ΔCt similar to that previously found in MDM from healthy donors demonstrating that lung and MDM expressed CFTR at similar levels (Fig 16C).

Bactericidal activity of CF lung macrophages against *P. aeruginosa* is impaired

With the aim to verify whether lung CF macrophages displayed defective bactericidal activity, I compared the ability of CF and non-CF macrophages to kill *P. aeruginosa* by antibiotic protection assay over a four hour time period. Lung macrophages were infected with Pa27853 and, following infection, intracellular live bacteria were detected at three time points: at the end of infection (t0), two (t2) and four hours after infection (t4) by the colony forming unit (CFU) method. Results from the analysis revealed that the median percentage of surviving bacteria in non-CF macrophages decreased from 66% (t2) to 36,6% (t4) (Fig 2). On the contrary, bacterial survival within CF lung macrophages didn't differ over time with a median survival of 98% and 92% two and four hours after infection respectively. Bacteria survival in CF macrophages was significantly higher than in non-CF cells four hours following infection (P=0,018) whereas no significant difference was observed at the early time point (P=0,297) although a greater fraction of surviving bacteria was recovered from CF cells as compare to non-CF. The survival advantage of *P. aeruginosa* in CF lung macrophages could not be explained by altered bacteria internalization because the fraction of intracellular live bacteria recovered at the end of infection (t0) was similar in non-CF (0.58% +/- 0.72) and CF (0.47 +/- 0.84) cells (figure 17).

These data extend to lung macrophages the defect previously observed in CF MDM (figure 17), i.e an impairment in the elimination of intracellular *P. aeruginosa* four hours after infection. Moreover, CF lung macrophages were more severely compromised in their bactericidal activity than CF MDM. Indeed the percentage of live intracellular bacteria in CF lung macrophages approached the 100% both two and four hours after infection, differently from CF MDM in which a progressive reduction over the time in the percentage of intracellular live bacteria was observed.

Overall our results, demonstrate that defective CFTR alters the bactericidal activity of CF macrophages.

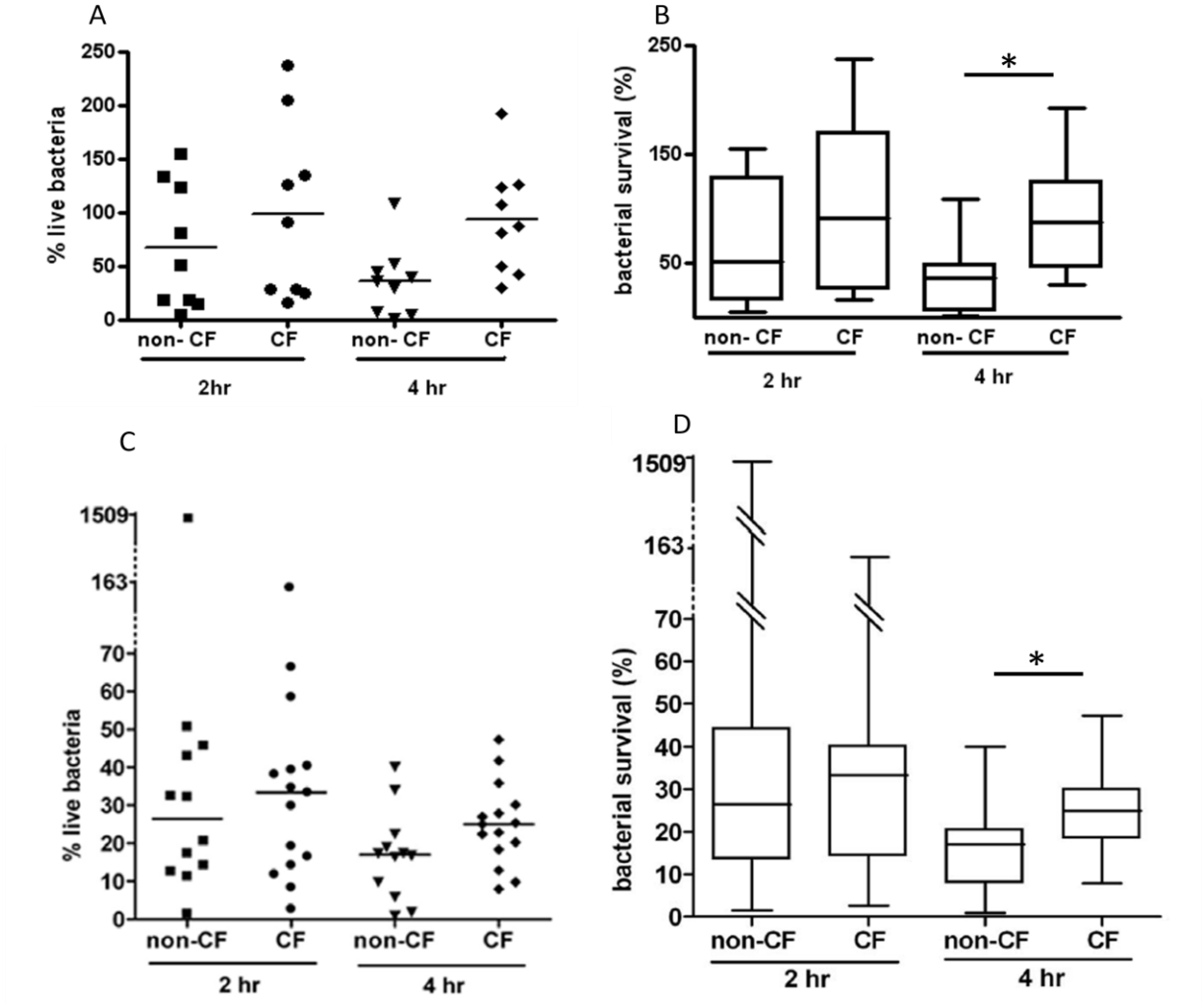

Figure 17. *P. aeruginosa survival in human macrophages isolated from non-CF and CF patients. Lung macrophages: A) Percentage of intracellular live bacteria rescued two (2 hr) or four (4 hr) hours following infection. Data were normalized to bacteria recovered at the end of infection (t0) set as 100%. Each symbol represents a single individual. The line is the median percentage of live bacteria. B) Box plots of data in A; the 25 and 75 percentiles, median, minimal and maximal values are represented. Statistical analysis (Mann Whitney non parametric test), non-CF vs CF 4 hours following infection P= 0.0188. MDM: C) Percentage of intracellular live bacteria rescued two (2 hr) or four (4 hr) hours following infection. Data were normalized to bacteria recovered at the end of infection (t0) set as 100%. Each symbol represents a single individual. The line is the median percentage of live bacteria.D) Box plots of data in C; the 25 and 75 percentiles, median, minimal and maximal values are represented. Statistical analysis (Mann Whitney non parametric test), non-CF vs CF 4 hours following infection P= 0.0359.*

P. aeruginosa infection induces oxidative burst in CF and non-CF human macrophages

It has been proposed that dysfunctional CFTR might affect the assembly and the activation of NADPH oxidase impairing ROS generation and bacterial killing. ROS in phagocytic cells are mainly produced by the NADPH oxidase 2 (NOX2) (Nauseef WM, 2004). NOX2 activity and the huge rise of oxygen consumption associated with ROS production (oxidative burst), have been widely addressed in neutrophils, and very few is known about it in human macrophages (Flannagan et al, 2009). Thus, as first I analyzed ROS production by human macrophages challenged with *P. aeruginosa*. The oxidative burst was monitored over time by infecting human non-CF MDM with Pa27583 in the presence of luminol. MDM from three non-CF individuals showed a rapid oxidative burst response that peaked at 20 minutes after bacterial infection (figure 18). Thereafter responses declined, reaching the basal level at 50 minutes. The specificity of the reaction was demonstrated by addition of DPI, an inhibitor of the NOX2 NADPH oxidase, that completely abolished the respiratory burst.

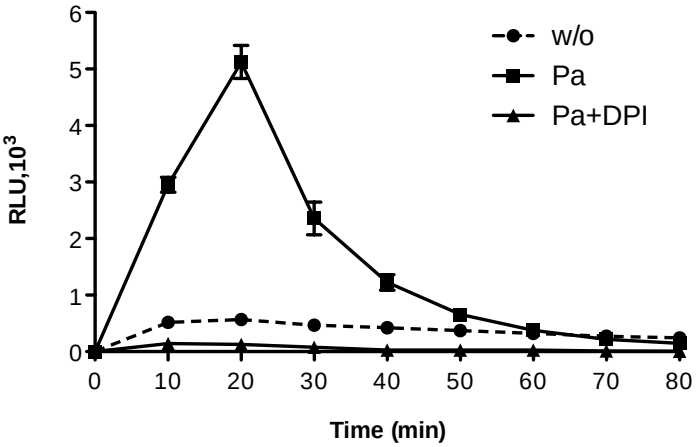

Figure 18. *Kinetic analysis of ROS production following P. aeruginosa infection of MDM as detected by chemioluniscence. Data are representative of three indipendent experiment performed in triplicate. Y axis indicates Relative Luminescence Unit (RLU). Values are Mean ± Std.*

Next, the intracellular production of ROS in CF and non-CF macrophages has been determined by loading the cells with the redox sensitive probe CM-H2DCFDA. *P. aeruginosa* infection of non-CF or CF MDM induced a significant increase in ROS production compared

to non-infected cells (figure 19A). The capability of macrophages to produce ROS was further addressed by an NADPH oxidase activator, PMA, which induced a similar increase of DCF fluorescence in non-CF (MFI, 728 +/- 208) and CF macrophages (MFI, 804 +/-390). Similarly, bacterial infection induced ROS production in CF lung macrophages although the increase was not statistically significant. In addition, similar intensities of DCF fluorescence were generated by *P. aeruginosa* infected non-CF and CF macrophages (figure 19B) suggesting that dysfunctional CFTR does not affect the oxidative burst capability of human macrophages.

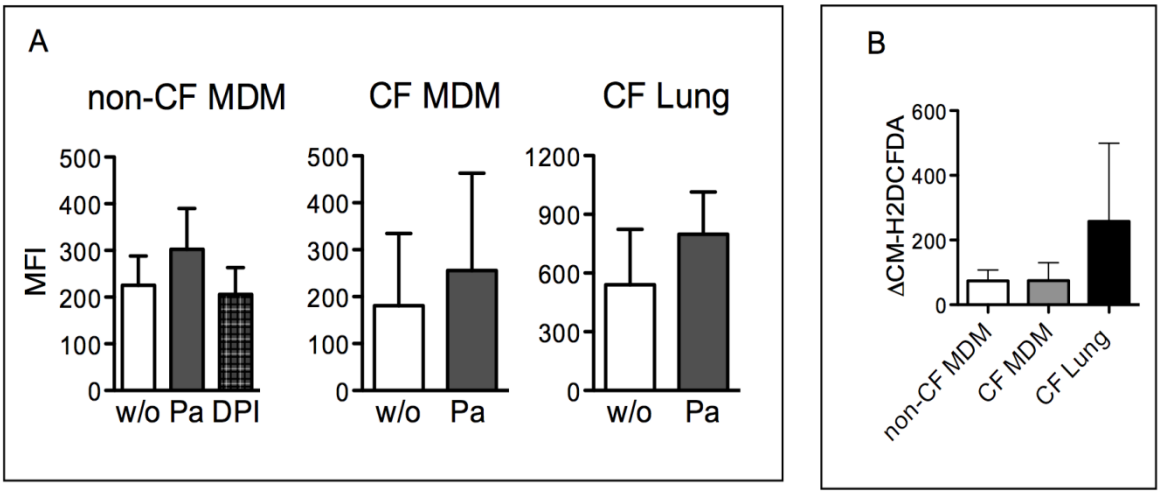

Figure 19. *Oxidative burst response of non-CF and CF human macrophages challenged with P. aeruginosa. Samples: non-CF MDM (N=8), Monocyte derived macrophages isolated from non CF subjects; CF MDM (N=6), Monocyte derived macrophages isolated from CF patients; CF Lung (N=3), lung macrophages isolated from CF patients.*

A: MDM or lung macrophages loaded with CM-H2DCFDA were infected with P. aeruginosa; the Mean Fluorescence Intensity (MFI) of non infected (w/o) or infected (Pa) macrophages was measured by flow cytometry. Values are Mean ± Std.

Significant differences between uninfected and infected cells were determined by Wilcoxon non parametric t-test: non-CF MDM non-infected vs infected, p= 0,0078; CF MDM non-infected vs infected , p= 0,0300; CF Lung=0.250.

B: Relative ROS production induced by P. aeruginosa was detected by the change in the fluorescence of CM-H2DCFDA. ΔCM-H2DCFDA Fluorescence was calculated using the following formula: ΔCM-H2DCFDA Fluorescence= MFI $_{infected}$ ¯ MFI $_{uninfected}$

Inhibition of NADPH oxidase increases intracellular *P. aeruginosa* survival in CF and non-CF macrophages

Having demonstrated that *P. aeruginosa* induced ROS production both in MDM and lung macrophages, we determined the contribution of ROS to bacterial killing by these cells.

Because ROS production peaked 20 min after infection and reached the basal level 30 min later, intracellular bacteria survival was monitored 2 hours from infection. Non-CF and CF MDM were pre-treated with DPI and infected with Pa27853. Then intracellular bacteria recovered from un-treated or treated cells were determined. Results obtained with MDM from non-CF or CF patients demonstrated that NADPH inhibition by DPI caused an increase in the number of surviving intracellular bacteria compared to untreated cells (figure 20A). Since DPI

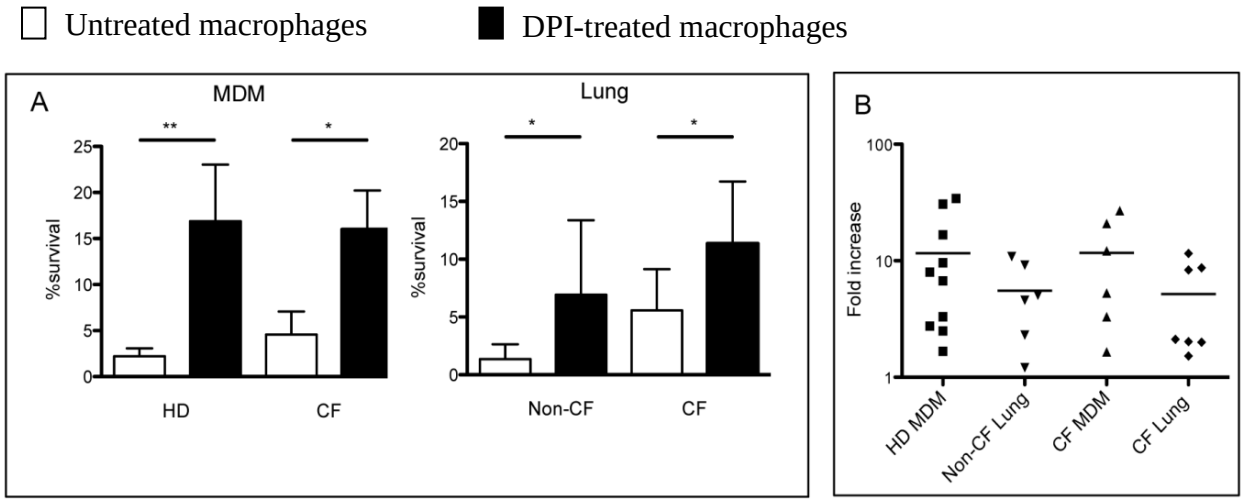

Figure 20. *Contribution of ROS to bacterial killing by macrophages isolated from non-CF or CF patients.*

*A) Percentage of intracellular live bacteria rescued from untreated (white bar) or DPI-treated (black bar) macrophages. Samples: MDM, monocyte derived macrophages from healthy donors (non-CF, N= 10) or CF patients (N=6); lung macrophages from non-CF (N=6) or CF (N=7) patients. *p< 0.05, **p<0.01, Wilcoxon non parametric t-test.*

B) Fold increase of intracellular live bacteria in DPI-treated macrophages versus untreated cells. Each symbol represents a single subject, the line is the mean fold increase.

does not distinguish between NADPH oxidase or iNOS (inducible nitric oxide synthase) activity, inhibition of iNOS was used to determine whether its activity contributed to bacteria

killing at this stage of infection. Pre-treatment of macrophages with L-NAME, a specific inhibitor of iNOS, had no effect on the bacterial killing by MDM. This result, in addition to previously published data demonstrating that bacterial stimuli are not sufficient to induce NO production in human MDM, strongly suggests that ROS production contributes to macrophages bacteria killing, at least in the first 2 hours following infection regardless CFTR function (Daigneault M et al, 2010).

Next, the effect of ROS inhibition in non-CF and CF lung macrophages bactericidal activity was also investigated (figure 20A). Similarly to MDM, non-CF and CF lung macrophages treated with DPI exhibited and increase of the bacterial survival.

Finally, the survival advantage of *P. aeruginosa* in DPI treated cells was quantified by determining the fold increase of live bacteria in DPI-treated *versus* un-treated cells (figure 20B). Any difference in the fold increase was observed between CF and non-CF macrophages both in MDM and lung macrophages. Overall, this data confirmed the importance of ROS in the elimination of *P. aeruginosa* by macrophages regardless their origin, MDM or lung, and CFTR function.

PART III: Cathepsins and matrix metalloproteinases (MMPs) affect bactericidal ability of macrophages

Inhibition of MMP and aspartate proteases in marcophages improves their bactericidal ability

The results obtained by the analysis of the oxidative mechanisms clearly demonstrated that they contribute to *P. aeruginosa* killing regardless CFTR activity. Besides oxidative mechanisms macrophagic proteases play a fundamental role in the destruction of the engulfed pathogens (Flannagan RS, 2009). Thus, due to the relevance of proteases in bactericidal activity of phagocytic cells, the aim of my study was to verify whether proteases are involved in the elimination of *P. aeruginosa* by human macrophages. To address this issue I used the human pro-monocytic cell line THP-1 that is a well established monocyte/macrophages model (Daigneault M et al, 2010). THP-1 monocytic cells have been differentiated *in vitro* into macrophages by PMA (Phorbol-12-myristate-13-acetate) treatment. Analysis of cell morphology, cell adhesion and expression of surface markers demonstrated that these cells closely resemble the phenotype of human MDM.

THP-1 cells were pre-treated with different protease inhibitors and infected with *P. aeruginosa*, strain PaO1. Subsequently the bacteria survival in un-treated or treated cells was determined by the antibiotic protection assay. Figure 21 outlined the experimental approach used for this part of the work. Specific inhibitors for each of main classes of proteases expressed by macrophages were used: cysteine proteases were inhibited by E-64, aspartate proteases by pepstatinA, serine proteases by pefabloc and metalloproteinases by EDTA.

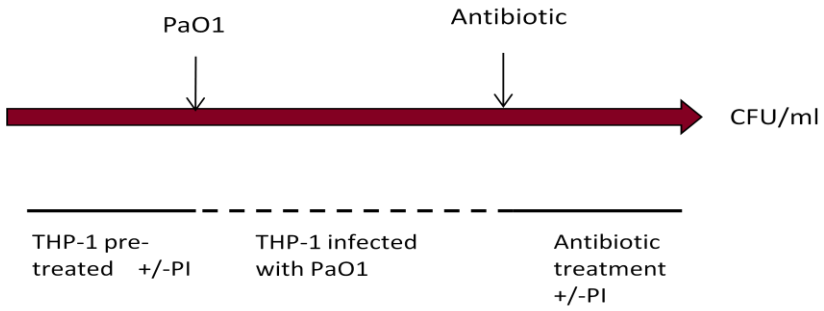

Figure 21. *Experimental scheme used to analyze protease inhibitor (PI) effect on bactericidal activity of THP-1 cells.*

Results of THP-1 infection assay demonstrated that the intracellular survival of *P.aeruginosa* was reduced by 85% when the cells were treated with a mixture containing all the inhibitors. Individual inhibitor testing revealed a statistical significant reduction of surviving intracellular bacteria in the presence of pepstatinA and EDTA (figure 22). Since EDTA is an unspecific metalloprotease inhibitor, to confirm the role of this class of proteases in the observed bacterial killing, the effect of GM6001 and phosporamidon, two specific MMP inhibitors, were tested (figure 23). Bacterial survival in cells pre-treated with both MMP inhibitors, was reduced compared to control cells confirming previous data, although the magnitude of the reduction was lower than that obtained by EDTA.

MMP inhibitors do not affect ROS production in THP-1 cells

Interestingly, ROS-mediated *P.aeruginosa* killing occurs within two hours following infection and protease inhibitors affect bactericidal activity of macrophages with the same kinetic. Thus, in order to establish whether metalloproteases influence ROS production, I determined the oxidative burst in THP-1 cells challenged with bacteria stimuli. To test this hypothesis intracellular ROS were measured by CM-H2DCFDA in macrophages treated or

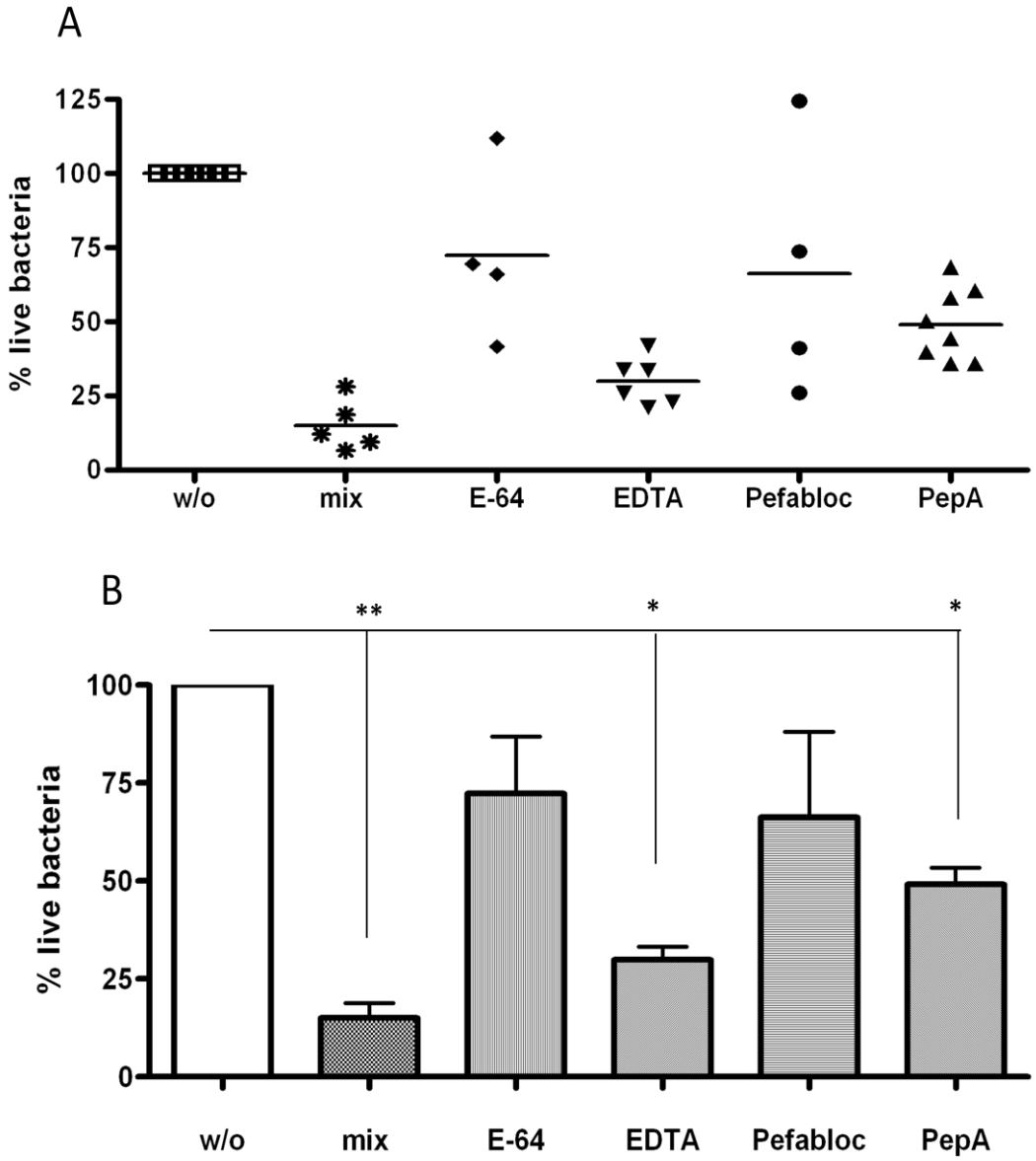

Figure 22. *P. aeruginosa survival in THP-1 cells treated with protease inhibitors.*

A) Percentage of live intracellular bacteria in THP-1 differentiated cells. Data were normalized to bacteria recovered from controls cells (w/o) set as 100%. Each symbol represents a single experiment. The line is the median percentage of live bacteria.

B)Summary data of percentage of live intracellular bacteria in THP-1 differentiated cells. Statistical analysis Kruskal-Wallis non parametric test.

(mix=E-64+EDTA+Pefabloc+PepstatinA).

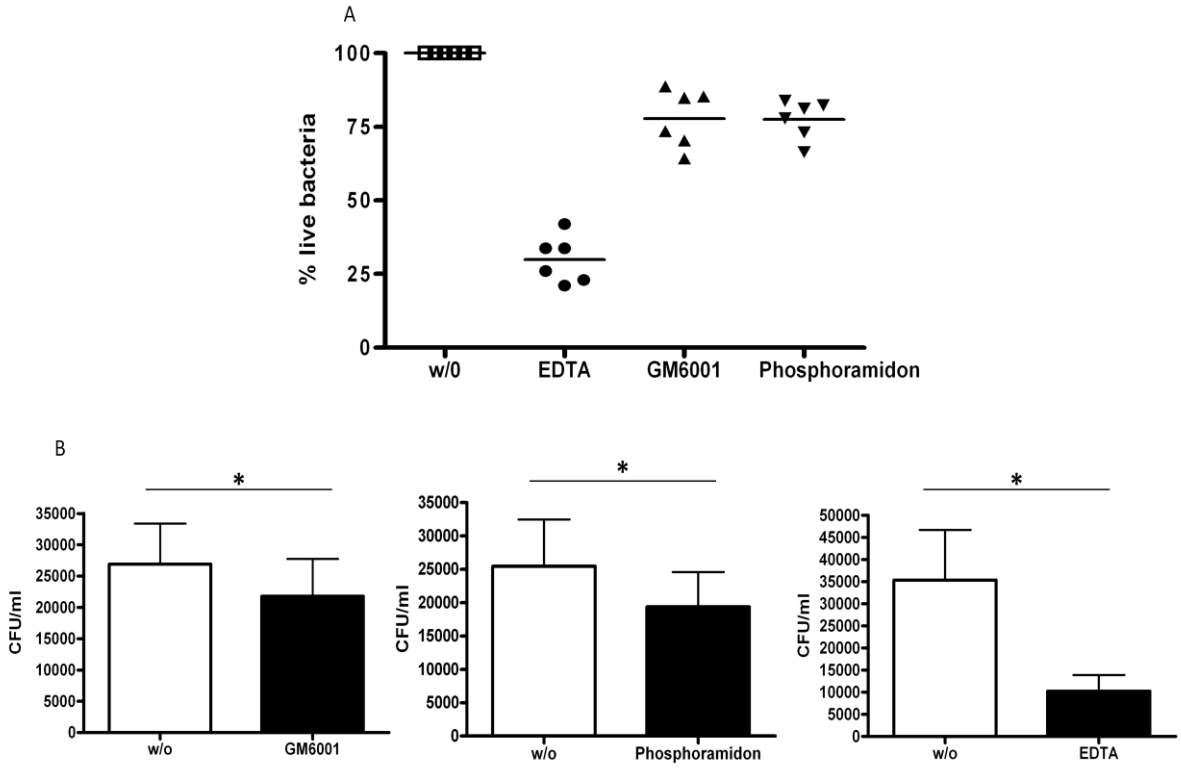

Figure 23. *P. aeruginosa survival in THP-1 treated with MMP inhibitors.*

A) Percentage of live intracellular bacteria in THP-1 differentiated cells. Data were normalized to bacteria recovered from controls cells (w/o) set as 100%. Each symbol represents a single experiment. The line is the median percentage of live bacteria.

B) Surviving bacteria within THP-1 differentiated cells; statistical analysis (Wilcoxon non parametric test): w/o vs GM6001 (N=6), P=0,0313; w/o vs Phpspsoramidon (N=6), P=0,0313; w/o vs EDTA (N=6), P=0,0313.

untreated with protease inhibitors and then challenged with fMLP or bacteria. fMLP, a formylated peptide of bacterial origin, in addition to be a potent inducer of phagocyte chemotaxis is also involved in generating antimicrobial agents such as nitric oxide (NO) and ROS (Klestadt D et al, 2005). NADPH oxidase inhibitor DPI completely blocked ROS production in challenged cells demonstrating that ROS detected in these experiments derived from NADPH oxidase acitvity (figure 24). The results obtained did not show significant difference in the signal between THP-1 cells treated with MMP inhibitors and untreated cells. This result suggests that protease inhibitors have no effect on induced ROS production thus

arguing against the possibility the increased bactericidal activity of treated macrophages are due to ROS.

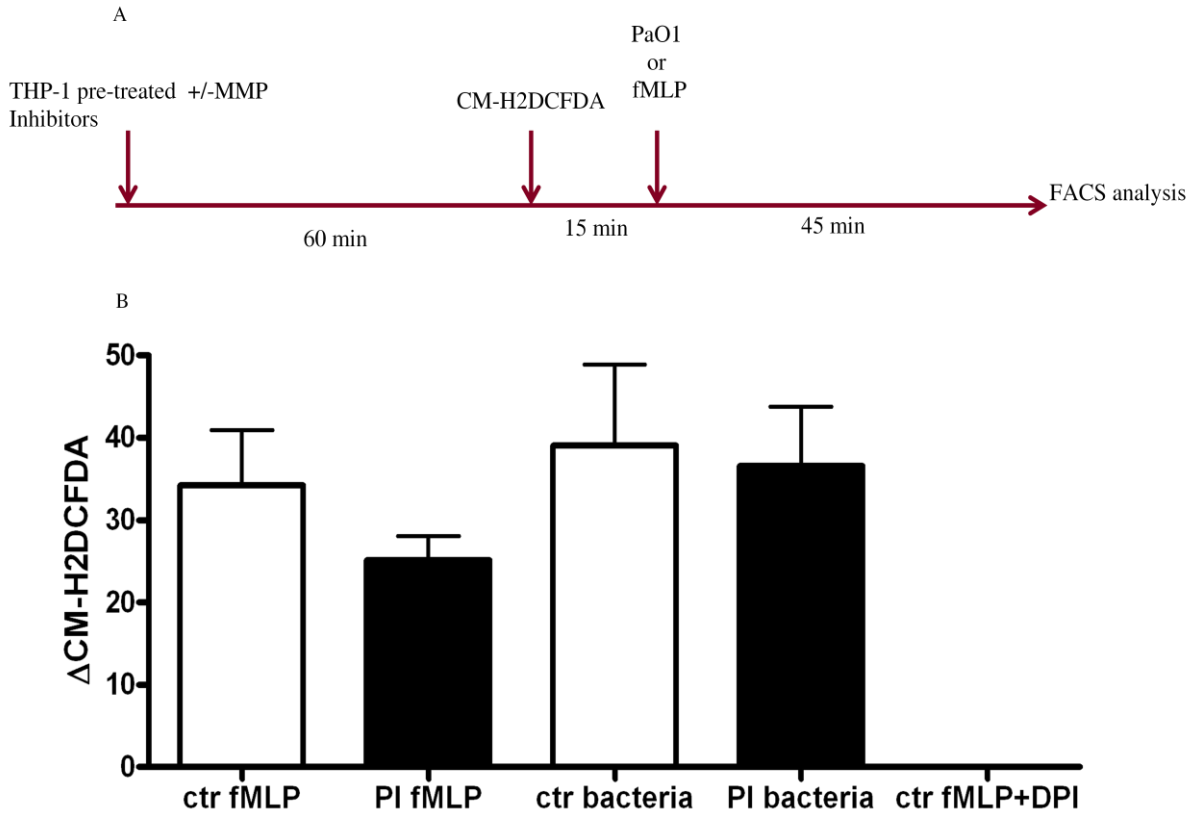

***Figure 24**. ROS production in THP-1 cells treated with MMP inhibitors.*

A) Experimental scheme used to analyze MMP inhibitor effect on ROS production by THP-1 cells following stimulation with PaO1 or fMLP.

B) THP-1 cells loaded with CM-H2DCFDA were infected with P. aeruginosa or stimulated with fFMLP; the Mean Fluorescence Intensity (MFI) of cells treated with MMP inhibitors (PI) or untreated (ctr) was measured by flow cytometry. Ctr (N=4), PI (N=4); values are Mean ± Std.

MMP inhibitors do not change lysosomal pH in THP-1 cells

One possible explanation of the result obtained with the protease inhibitors is that they may act directly by decreasing the lysosome pH, which contributes to *P. aeruginosa* killing. In order to test this hypothesis THP-1 cells treated or un treated with MMP inhibitors, were infected with PAO1 labeled with BCECF-AM, one of most widely used fluorescent indicator

for intracellular pH (Chow S, 2001). In detail, the indicator BCECF-AM is membranepermeant due to its acetoxymethyl (AM) ester groups. Once inside the cell, the non nonfluorescent BCECF-AM is hydrolyzed by nonspecific cellular esterases to yield the free fluorescent dye. This form of the indicator has different emission spectra and taking the ratio of the two resulting emissions gives a signal that is proportional to pH and independent of cellular dye content. The pH value of the samples has then been interpolated from the calibration curve done by incubating PaO1 labeled in potassium buffers at known pH.

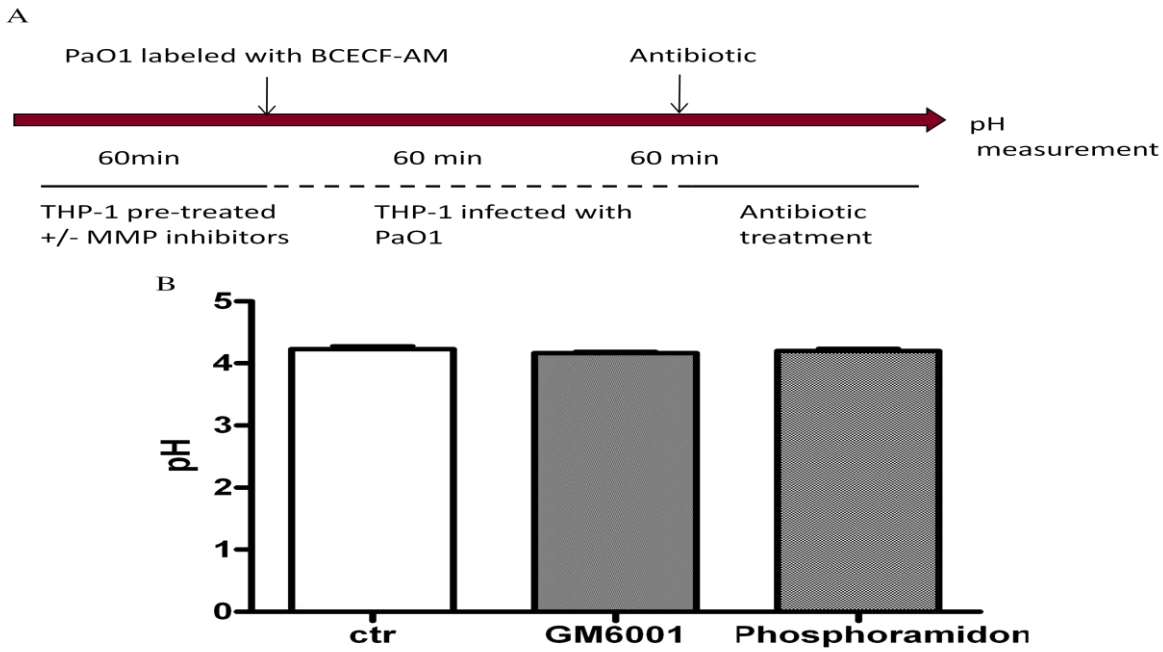

Figure 25. *Phagolysosome pH in THP-1 cells treated with MMP inhibitors.*

A) Experimental scheme used to analyze MMP inhibitor effect on phagolysosome pH in THP-1 cells.

B) THP-1 cells were infected with PaO1 labeled by BCECF-AM; the pH values of cells treated with MMP inhibitors or untreated (ctr) was interpolated from the calibration curve . Ctr (N=5), GM6001(N=5), Phosphoramidon (N=5); values are Mean ± Std.

This approach fail to reveal any difference in the lysosmal pH between MMP inhibitors treated cells and untreated cells (figure 25), suggesting that lysosomal pH is not affected by MMP inhibitors. To further confirm this result cytosolic pH has been determined using the same indicator to directly label the cells. The cytosolic pH values have been interpolated from the calibration curve done by incubating labeled cells treated with the ionophore nigericin in

potassium buffers at known pH. The pH value was approximately 7 both in THP-1 treated with MMP inhibitors and untreated cells, confirming that pH values obtained by labeling PaO1 indicated lysosomal pH values.

Discussion

Lung disease, characterized by chronic infection and inflammation, is the major cause of morbidity and mortality in CF patients (Davis PB, 2001). For a long time the main cause of CF lung pathology has been ascribed to dysfunctions in the defence mechanisms mediated by airway epithelial cells. Indeed CFTR mutations in epithelial cells altering chloride transport lead to air liquid interface dehydration, thick mucus deposition and impaired muco-ciliary clearance (Matsui H et al, 1998). Recently, a growing body of evidences suggest that other innate immune cells such as neutrophils and macrophages are directly affected by CFTR dysfunction, thus contributing to infectious pathology in CF lung (Ratner D and Mueller C, 2012). In particular alterations in macrophage bactericidal activity and secretion of proinflammatory cytockines have been proposed to contribute to hypersuceptibility to bacteria infections and exaggerated inflammation in CF patients. In this context Bruscia et al reported that LPS-stimulated CF macrophages secrete higher levels of pro-inflammatory cytokines compared to WT macrophages as a result of abnormal trafficking of TLR4 (Bruscia EM et al et, 2009; Bruscia EM et al et, 2011).

In addition it has been demonstrated that *cftr*-deficient murine alveolar macrophages display a reduced ability to kill *P. aeruginosa* and this defect has been confirmed in murine macrophages carrying the most common CFTR mutations F508del and G551D (Di et al., 2006; Deriy et al, 2009; Zhang et al, 2010).

In order to study the contribution of CFTR activities of human macrophages we first verified the expression of CFTR in these cells demonstrating that CFTR is mainly localized to surface plasma membranes of human monocyte derived macrophages (MDM) where it acts as a cAMP-dependent chloride channel (Del Porto et al, 2011). Next I focused the study on the effect of CFTR mutations on bactericidal activity of human macrophages.

Results obtained by antibiotic protection assays revealed a statistically significant increase in the percentage of live intracellular bacteria in CF macrophages. Both macrophages differentiated *in vitro* or isolated from the lung exhibited a reduced capability to kill bacteria four hours after infection compared to control cells extending to human macrophages previous evidences from the murine models. Moreover, the bactericidal defect was more pronounced in lung macrophages than MDM as demonstrated by the comparative analysis of the reduction of intracellular survival of *P. aeruginosa* over time. Indeed the median percentage of live intracellular bacteria approached 100% in CF lung macrophages two and

four hours after infection differently from CF MDM in which a survival of 33,34 % and 25% was observed two and four hour after infection, respectively. These data suggested that CF macrophages are intrinsically defective in their bactericidal activity regardless environmental influences. Accordingly, defective bactericidal activity at the same time point (t4) was observed in CF MDM and CF lung macrophages compared to controls cells supporting the role of CFTR in the antimicrobial activity of human macrophages and suggesting that CFTR dysfunctions affect the same microbicidal mechanism in phagocytes isolated from different body sites.

It has been proposed that dysfunctional CFTR might affect both oxidative and non oxidative mechanisms of bacterial killing by macrophages including ROS generation mediated by NADPH oxidase and lysosomal pH (Zhang et al, 2010; Di et al, 2006). Due to the lack of data concerning the generation of oxidative burst in human macrophages following *P. aeruginosa* infection, first I verified the production of ROS in macrophages challenged with Pa27583 by luminol chemioluminescence. This method allowed to demonstrate that bacterial infection induced ROS production in human macrophages with a maximum peak at 20 minutes post infection and completion within one hour, a kinetic similar to that previously reported for human neutrophils. The dependence of the observed ROS on NADPH oxidase activity was demonstrated by the inhibition of ROS generation by DPI.

The role of ROS in *P.aeruginosa* killing has be reported in human neutrophils, instead less is known about macrophages (Sadikot RT et al, 2005; Mishra et M al, 2012). Thus having demonstrated the ability of macrophages to generate ROS following bacterial infection, I analysed the contribution of ROS to bactericidal activity of these cells against *P. aeruginosa*. Inhibition of ROS production in human macrophages by DPI led to a significant increase in the intracellular bacteria survival in both MDM and lung macrophages from CF as well as non-CF subjects. Quantification of survival advantage of *P. aeruginosa* in DPI treated cells revealed a mean increase of 10 fold and 6 fold in MDM and lung macrophages, respectively regardless the presence of functional CFTR. This data indicate that ROS represent a relevant effector of the macrophages defence against *P. aeruginosa* infection and that this pathway is preserved in CF human macrophages. This latter finding is further supported by the evidence that measurement of ROS production in macrophages challenged with *P. aeruginosa,* or stimulated with PMA, by CM-H2DCFDA does not reveal any difference between CF and non-CF cells confirming that CFTR dysfunctions do not affect the oxidative burst in human

macrophages. Similarly, lack of intrinsic defects in ROS generation has been recently reported for CF human neutrophils through a detailed analysis of the oxidative output in response to receptor-linked and particulate stimuli (McKeon DJ et al, 2010).

The evidence that defective bacterial killing in human CF macrophages could not be ascribed to an impairment of ROS generation is at variance with results reported on murine alveolar macrophages. Indeed Zhang and collaborators have previously demonstrated that NADPH oxidase fails to assemble and activate ROS production in CF murine macrophages thus compromising the ability of these cells to kill *P. aeruginosa*. This defect was attributed to altered ceramide metabolism as a consequence of pH alteration in intracellular vesicles affecting the activity of enzymes involved in ceramide metabolism (Zhang et al, 2010). All together these data suggest that CFTR dysfunctions have specie specific consequences on macrophage functions. This might explain the earlier defect in bactericidal activity observed in murine CF macrophages compared to human CF cells. Indeed an impairment of *P. aeruginosa* killing by *Cftr*-deficient murine macrophages was observed as soon as one hour after infection and it was maintained after (Di et al, 2006), whereas in this thesis human CF macrophages did not display an early defects but are significantly impaired in their bactericidal activity only later over infection.

Having demonstrated that human CF macrophages are impaired in their bactericidal activity against *P. aeruginosa* and that this defect is not due to defective ROS production, the analysis of others microbicidal mechanisms employed by phagocytes to kill intracellular bacteria has been undertaken. Among microbicidal mechanisms of phagocytes cathepsins, together with other lysosomal enzymes, play a fundamental role in the eradication of intracellular bacteria (Flannagan RS et al, 2009). Although many of MMP functions had been thought to occur extracellularly or on the cell surface, it has been reported that macrophage MMP-12 has an intracellular direct antimicrobial activity (Houghton AM et al, 2009). Thus the contribution of lysosomal enzymes to bactericidal activity of THPI-1 cells against *P.aeruginosa* has been analyzed. Unexpectedly, the inhibition of protease activity in THP-1 cells by protease inhibitors decreased the intracellular survival of *P. aeruginosa.* Particularly the inhibition of MMPs and aspartate proteases induced a statistically significant reduction in the percentage of live intracellular bacteria compared to control cells suggesting that MMP and aspartate protease inhibitors improve *P. aeruginosa* killing by THP-1 cells. Thus, it appears that proteases can inhibit the bactericidal ability of human macrophages possibly by acting in one

or more pathways of microbial killing. At the moment I can exclude that proteases act on oxidative dependent mechanisms or on lysosomal pH, at least in THP-1 macrophages. Alternatively, it may be that protease inhibitors block the bacteria proteases making them less virulent and more susceptible to macrophage killing. This hypothesis is under investigation by using protease deficient *P. aeruginisa* strains.

Overall, although further studies are required to highlight the mechanisms by which protease inhibitors act, the possibility to use these compounds to improve macrophage microbicidal activity may be relevant for the development of new therapeutic strategies for the treatment of CF lung disease and possibly other infective disease.

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