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Review

Extracellular pH and lung infections in cystic fibrosis

María Macarena Massip-Copiz, Tomás Antonio Santa-Coloma*,[1]

Laboratory of Cellular and Molecular Biology, Institute for Biomedical Research (BIOMED UCA-CONICET), The National Scientific and Technical Research Council (CONICET), and School of Medical Sciences, The Pontifical Catholic University of Argentina (UCA), Buenos Aires, Argentina

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ABSTRACT

Cystic fibrosis (CF) is an autosomal recessive disease caused by *CFTR* mutations. It is characterized by high NaCl concentration in sweat and the production of a thick and sticky mucus, occluding secretory ducts, intestine and airways, accompanied by chronic inflammation and infections of the lungs. This causes a progressive and lethal decline in lung function. Therefore, finding the mechanisms driving the high susceptibility to lung infections has been a key issue. For decades the prevalent hypothesis was that a reduced airway surface liquid (ASL) volume and composition, and the consequent increased mucus concentration (dehydration), create an environment favoring infections. However, a few years ago, in a pig model of CF, the Na^+/K^+ concentrations and the ASL volume were found intact. Immediately a different hypothesis arose, postulating a reduced ASL pH as the cause for the increased susceptibility to infections, due to a diminished bicarbonate secretion through CFTR. Noteworthy, a recent report found normal ASL pH values in CF children and in cultured primary airway cells, challenging the ASL pH hypothesis. On the other hand, recent evidences revitalized the hypothesis of a reduced ASL secretion. Thus, the role of the ASL pH in the CF is still a controversial matter. In this review we discuss the basis that sustain the role of CFTR in modulating the extracellular pH, and the recent results sustaining the different points of view. Finding the mechanisms of CFTR signaling that determine the susceptibility to infections is crucial to understand the pathophysiology of CF and related lung diseases.

1. Introduction

Cystic fibrosis (CF), or mucoviscidosis, is an autosomal recessive disease caused by mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene (Quinton, 2010; Riordan, 2008). It is characterized by severe pancreatic and lung dysfunctions that eventually lead to organ failure (Wiencek and Lo, 2018). Besides these organs, the abundant secretion of dehydrated mucins in CF (Henderson et al., 2014) affects the digestive and reproductive organs, and glandular ducts (Wang et al., 2014; Wiencek and Lo, 2018). The pulmonary damage is the major cause of morbidity and mortality in CF patients (Savant and McColley, 2017). A vicious cycle of persistent inflammation and infections affects severely the pulmonary parenchyma (Nichols and Chmiel, 2015). The main respiratory pathogens found in CF patients are *Pseudomonas aeruginosa* (Hoiby, 2011; Mauch et al., 2018), *Staphylococcus aureus* (Schwerdt et al., 2018; Wong et al., 2013), and *Burkholderia cepacia* (Regan and Bhatt, 2016). Eventually lung transplantation is needed for CF patients with end-stage lung disease (Snell et al., 2017).

Considering that an acidic environment might constitute a key characteristic allowing bacterial establishment (Berkebile and McCray, 2014; Coakley and Boucher, 2001; Lardner, 2001; Pezzulo et al., 2012), in this review we will discuss the basis that withstand a role of CFTR in modulating the extracellular pH (pHe), and recent findings sustaining or challenging the pHe hypothesis.

2. CFTR channel structure and function

In 1989 the *CFTR* gene was cloned (Riordan et al., 1989). *CFTR* mutations and the consequent CFTR channel failure produce the complex CF phenotype (Lim et al., 2017; Riordan, 2008). More than 2000 different mutations have been described for the *CFTR* gene, being the ΔF508 mutation the most common (Veit et al., 2016). The *CFTR* gene codifies for an ion channel which is a member of the superfamily of ABC (ATP Binding Cassette) transporter proteins. This transmembrane glycoprotein is formed by two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs) and a regulatory domain (R), which is unique among ABC transporters (Callebaut et al., 2017; Liu

* Corresponding author at: Laboratory of Cellular and Molecular Biology (LBCM), Institute for Biomedical Research (BIOMED), The Pontifical Catholic University of Argentina (UCA), Alicia Moreau de Justo 1600, 3rd Floor, Bldg. San José, C1107AFF, Buenos Aires, Argentina.
E-mail address: tomas_santacoloma@uca.edu.ar (T.A. Santa-Coloma).
[1] www.uca.edu.ar/iib.

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et al., 2017). The R domain, primarily activated by PKA phosphorylation and by other serine/threonine and tyrosine kinases (PKC, c-Src), controls the opening of the channel, and the two NBDs control channel gating (Rogan et al., 2011). Apart from acting as an ATP-gated chloride (Cl^-) channel, CFTR participates directly or indirectly in the transport of bicarbonate (HCO_3^-) (Kunzelmann et al., 2017), glutathione (GSH) (Kogan et al., 2003), and ATP (Egan, 2002). Because of the impaired Cl^- and HCO_3^- transport through CFTR in CF, secretions have an altered salt and fluid composition, characteristic of CF patients. Besides this, many cellular processes and signaling pathways are dysregulated in CF cells as a consequence of the CFTR deficiency, such as apoptosis (Soleti et al., 2013), autophagy (Mayer et al., 2013; Nakahira et al., 2014; Villella et al., 2013), redox balance (Ziady and Hansen, 2014), inflammation (Cohen-Cymberknoh et al., 2013; Giddings and Esther, 2017), innate and adaptive immunity (Bruscia and Bonfield, 2016; Hartl et al., 2012; Ralhan et al., 2016), mitochondrial functions (Valdivieso and Santa-Coloma, 2013), and mucin expression (Gonzalez-Guerrico et al., 2002; Kreda et al., 2012).

3. CFTR-dependent genes

After the *CFTR* was cloned most studies focused in the extracellular, non-genomics effects of the *CFTR* failure. We instead focused on possible genomic effects and hypothesized that the complex CF phenotype should be the result of a net of genes under CFTR regulation. By using differential display to test this hypothesis, we found several genes that responded to modulators of CFTR expression such as TPA (Cafferata et al., 1996) and IL-1β (González-Guerrico, 2001; González Guerrico et al., 1998). Later we applied a more specific strategy using CFTR inhibitors to find genes that specifically responded to the CFTR channel activity (González-Guerrico, 2001; González Guerrico et al., 1999). Some were further characterized, including *c-Src* (Gonzalez-Guerrico et al., 2002; Massip-Copiz et al., 2017; Massip Copiz and Santa Coloma, 2016), which in turn regulates MUC1 expression (Gonzalez-Guerrico et al., 2002), *CISD1*, a mitochondrial protein encoded in the nucleus with a yet ill-defined mitochondrial function (Taminelli et al., 2008), and *MTND4*, a mitochondrial protein encoded in the mitochondrial DNA, essential for the assembly and activity of the mitochondrial Complex I (Valdivieso et al., 2012, 2007). In parallel, we found that *CFTR* was upregulated in T84 cells by IL-1β (Cafferata et al., 2000a) via NF-kB (Cafferata et al., 2000b, 2001). Similar NF-κB dependency was later confirmed by Edelman´s laboratory using lung Calu-3 cells (Brouillard et al., 2001). Other laboratories also found *CFTR*-dependent genes by using microarrays (Galvin et al., 2004; Srivastava et al., 1999, 2002; Xu et al., 2003), although without further characterization of these genes.

The CFTR signaling intermediaries in the pathways that eventually lead to the expression of CFTR-dependent genes are largely unknown, with the exception of c-Src→MUC1 and IL-1β→c-Src (Gonzalez-Guerrico et al., 2002; Massip-Copiz et al., 2017; Massip Copiz and Santa Coloma, 2016), and Cl^- itself, which acts as a second-messenger for CFTR (Valdivieso et al., 2016; Valdivieso et al., 2017), inducing the expression and secretion of IL-1β (Clauzure et al., 2017, 2014). This cytokine in turn starts an autocrine (Sporn and Roberts, 1985) positive feedback loop that produces a chronic proinflammatory state in cultured CF IB3-1 cells (Clauzure et al., 2017, 2014), driving the reduction of the mitochondrial Complex I (mCx-I) activity (Valdivieso et al., 2012; Valdivieso and Santa-Coloma, 2013) and triggering oxidative stress (Clauzure et al., 2014; Massip-Copiz et al., 2017). Besides IL-1β and IL-1β-dependent genes, intracellular Cl^- regulates many other genes, among them *GRLX5* (Valdivieso et al., 2016) and *RPS27* (Valdivieso et al., 2016, 2017). We also found that CFTR→Cl^-→IL-1β regulate the expression of EGFR ligands; in particular, epiregulin (EREG) is upregulated through the IL-1β autocrine loop (Massip-Copiz et al., 2018). Noteworthy, it has been shown recently that IL-1β also stimulates CFTR mediated fluid transport in control but not in *CFTR*(-/-) swine trachea (Luan et al., 2017, 2014). In consequence, IL-1β turned out to be a very interesting CFTR- and Cl^--dependent gene.

The altered expression of this complex net of CFTR-dependent effectors and genes modulates at least the sterile proinflammatory and oxidative stress background, which is chronic, intrinsic to CF cells, and may contribute to the high susceptibility to infections. Therefore, the regulation of the pHe, the ASL secretion, and other phenotypic characteristics of CF, should not be seen only as the result of nongenomic effects.

4. Several hypotheses tried to explain the high susceptibility to infections in CF

An initial hypothesis considered that the increased susceptibility to infections in CF was due to abnormal mucus secretion (Alhadeff, 1978). With the years the idea was changing towards the hypothesis of an abnormal fluid and electrolyte transport (Quinton, 1989). Thus, since the *CFTR* was cloned by Riordan near three decades ago (Riordan et al., 1989), two different hypotheses attempted to explain the high susceptibility to infections in CF (Wine, 1999). The “high salt” hypothesis postulated that an increased NaCl concentration in the airway surface liquid (ASL), as occurred in the sweat duct (Quinton, 1983, 1986; Wine, 1999), inhibited antimicrobial defenses (Smith et al., 1996; Zabner et al., 1998). This was based on the observation that inoculated bacteria proliferated in CF conditioned medium but not in normal medium from control cells, and that diluted CF medium recovered the bactericidal properties; by the contrary, addition of NaCl to medium from control cells allowed bacteria to grow (Smith et al., 1996).

On the other hand, the “too little salt”/dehydration or low airway surface liquid (ASL) volume (water) hypothesis postulated that in the absence of normal CFTR activity the Cl^- secretion is reduced together with an increased Na^+ resorption (Boucher, 2007; Donaldson and Boucher, 2007; Matsui et al., 1998). It was proposed that CFTR normally inhibits the epithelial Na^+ channel (ENaC) activity, and that the absence of functional CFTR causes its hyperactivity, reduces Na^+/H^+ transport by electroneutral exchangers (NHE3), and consequently Na^+ hyperabsorption occurs (Gawenis et al., 2003; Mall et al., 1999). Interestingly, it has been proposed that ENaC hyperactivity is secondary to a reduced ASL pH in CF, which is sensed by SPLUNC1, a pH sensitive regulator of ENaC (Garland et al., 2013). This Na^+ hyperabsorption is thought to result in a decreased net H_2O transport, producing ASL dehydration and mucus concentration, contributing to the susceptibility to infections (Althaus, 2013; Kunzelmann et al., 2017).

However, no evidences of Na^+ hyperabsorption was found in sweat glands and, by the contrary, others found increased salt concentration in the CF ASL (discussed in (Kunzelmann et al., 2017)). In addition, in a *CFTR* knockout pig model developed by Michael Welsh’s team (Rogers et al., 2008a, b), in which bacterial infections occurred spontaneously, it was found later that the periciliary liquid depth (PCL) was not reduced. They also reported that no difference was found in Na^+ and K^+ concentrations in the ASL (Pezzulo et al., 2012). These results challenged the ASL volume/dehydration and Na^+ hyperabsorption hypothesis. As a consequence, a different hypothesis arose, based in previous reports (Coakley and Boucher, 2001; Coakley et al., 2003; Lardner, 2001; Luckie et al., 2001; Ojoo et al., 2005; Poulsen et al., 1994; Smith and Welsh, 1993; Song et al., 2006) and new insights (Abou Alaiwa et al., 2014; Borowitz, 2015; Cooper et al., 2013; Gelfond et al., 2017; Kunzelmann et al., 2017; Pezzulo et al., 2012; Shah et al., 2016; Shamsuddin and Quinton, 2014; Tang et al., 2016), which sustained that a reduced bicarbonate secretion through CFTR determines a low pH in the epithelial surface liquid, and that this low pH favors infections (Pezzulo et al., 2012). Yet, Schultz et al., in a recent work, contradicted these results suggesting that there is no difference in the ASL pH between CF and non-CF children, or in primary cultured cells (Schultz et al., 2017). McShane et al. previously made similar observations (McShane et al., 2003). Both concluded that CFTR may not

play a significant role in the ASL pH in vivo.

Meanwhile, as it will be discussed later, recent evidences using synchrotron X-ray imaging found increased secretion in the ASL induced by bacteria, flagellin or IL-1β in tracheal sections of swine, an effect that fails in *CFTR* (-/-) animals (Luan et al., 2017, 2014), or in vitro, under CFTR modulation (Shan et al., 2012), reinforcing the hypothesis of a diminished fluid secretion and consequently dehydration and impaired mucus clearance in CF (Button et al., 2012; Henderson et al., 2014; Knowles and Boucher, 2002; Quinton, 1989).

5. Channels and transporters involved in regulation of extracellular pH

A very important aspect of cellular homeostasis is the maintenance of the extracellular and intracellular pH (pHe and pHi). Due to the intrinsic complexity, many mechanisms are involved in pH regulation. Cells keep their acid-base regulation by using many metabolic enzymes, transporters and pH sensors. Their activities produce several acid-base equivalent species, including proton (H^+), lactate and carbon dioxide (CO_2) (Sanhueza et al., 2016), which are transported through plasma membrane (Seifter and Chang, 2017). The most important transporters that mediate pH homeostasis are (Fig. 1): Na^+/H^+ exchangers (NHEs) (Orlowski and Grinstein, 2011), vacuolar H^+ ATPases (V-ATPase) (Casey et al., 2010), H^+/K^+ ATPases (Gillies et al., 2004), Na^+/HCO_3^- transporters (NBCs) (Alka and Casey, 2014), and the bidirectional monocarboxylate transporters (MCTs/SCL16), particularly MCT1-4, that regulate the intracellular content of lactate (Halestrap, 2013a, b). The regulation of pHi has been extensively reviewed and will not be further considered here (Boron, 2004; Ruffin et al., 2014). Alterations of these transport systems, may produce not only an imbalance in the pHi but also an acidic pHe, favoring infections (Pezzulo et al., 2012). One key component in the pHe regulation is the HCO_3^- secretion, as it will be discussed below.

6. Bicarbonate transport

HCO_3^- is a very important electrolyte that takes part in pH regulation together with H^+ and lactate. Bicarbonate transporters and carbonic anhydrases are responsible for the regulation of HCO_3^- concentration in cells and their microenvironment (Fig. 1). More than 14 different bicarbonate transporters have been described and they are classified in two families: SLC4A and SLC26 A (SLC, Solute Carrier). The SLC4A family has 10 members, 9 of which are HCO_3^- transporters. They function as electroneutral Na^+-independent Cl^-/HCO_3^- exchangers (in particular AE1-3), electroneutral Na^+-coupled HCO_3^- co-transporters (NBCn1, NDCBE, NBCn2, and SLC4A9) or electrogenic exchangers Na^+-coupled HCO_3-cotransporters (NBCe1 and NBCe2) (Alka and Casey, 2014). The SLC26 A family has 5 members involved in HCO_3^- transport that may function as Na^+-independent electroneutral/electrogenic anion exchangers or as anion channels. SLC26A3, SLC26A4 (pendrin), and SLC26A6 are reported to be electroneutral and SLC26A7 and SLC26A9 are electrogenic (Alka and Casey, 2014). Apart from bicarbonate transporters, anion channels and metal transporters can also move bicarbonate. Besides CFTR (Borowitz, 2015), the Ca^{++}-activated anion channels bestrophin (Yu et al., 2010) and anoctamin 1 (Jung et al., 2013), and the GABA and glycine receptors/anion channels (Prescott, 2015) also have permeability to HCO_3^- (Alka and Casey, 2014). ZIP8 (SLC39A8) and ZIP14 (SLC39A14) are electroneutral symporters which transport divalent metal and HCO_3- (Nebert et al., 2012). A detailed description of HCO_3- transport in cell physiology and disease has been previously described (Alka and Casey, 2014).

7. CFTR and bicarbonate

The association between CFTR and HCO_3^- has been studied extensively in CF, and it has been recently reviewed (Kunzelmann et al., 2017). The CFTR contribution to pH regulation was reported in 1994 by

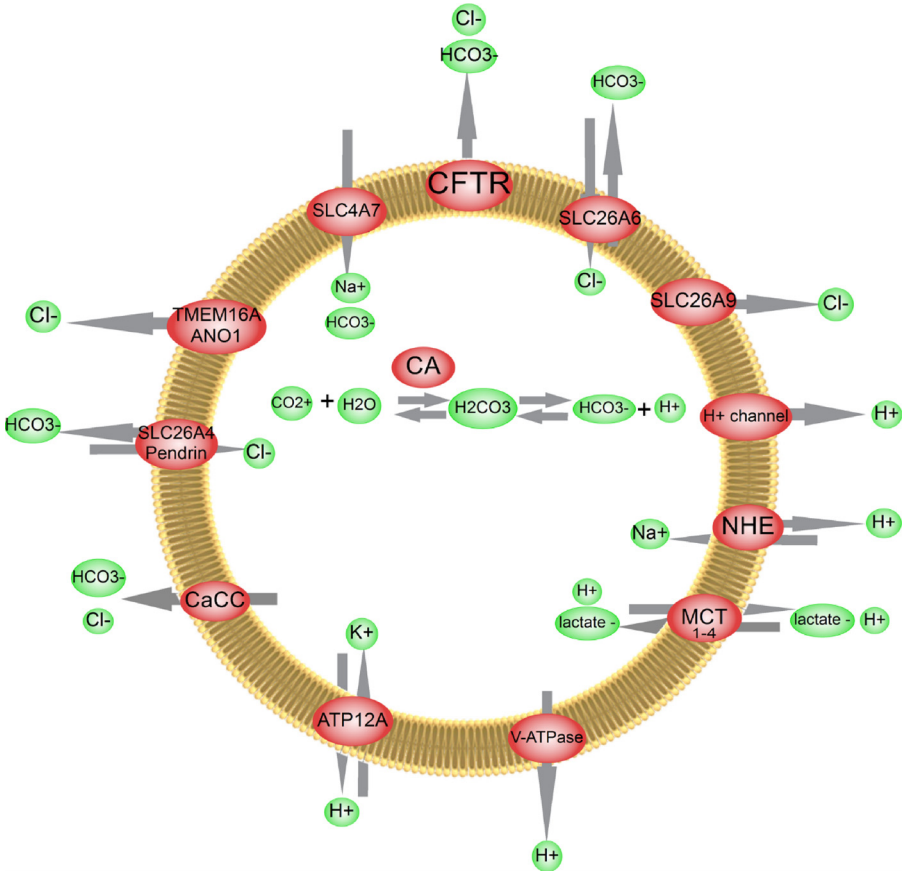

Fig. 1. pHe homeostasis. The figure summarizes the different channels and transporters involved in regulation of the extracellular pH (pHe): H^+ channels, Na^+/H^+ exchangers (NHEs), vacuolar H^+ ATPases (V-ATPase), H^+/K^+ ATPases, Na^+/HCO_3^- transporters (NBCs), and monocarboxylate transporters (MCTs/SCL16), particularly MCT1-4, that regulate the intracellular content of lactate. We focused principally on HCO_3^- transporters that interact with CFTR: NBCn1/SLC4A7, SLC26A6 and SLC26A4. Another member of SLC26 family, SLC26A9, also regulates CFTR activity. Among Ca^{2+}-activated chloride channels (CaCC) anoctamin-1 (ANO1/TMEM16 A) have a cellular crosstalk with CFTR and regulate its activity. Carbonic anhydrases (CA), which generate HCO_3^-, are also included. The figure was built by using the software Pathway Studio 10 (Elsevier).

Poulsen and colleagues using cultured NIH/3T3 fibroblasts and C127 mammary epithelial cells transfected with wild-type CFTR or ΔF508-CFTR (Poulsen et al., 1994). Since then, other laboratories have studied the effect of impaired CFTR function on extracellular acidification. Thus, a correlation between lack of CFTR and reduced HCO_3^- secretion was seen in primary cultures of surface bronchial and tracheal epithelial cells from humans and pigs (Ostedgaard et al., 2011), and in a variety of other model systems (Abou Alaiwa et al., 2014; Kunzelmann et al., 2017; Pezzulo et al., 2012; Shan et al., 2012; Tang et al., 2016). Under elevated cAMP conditions that activate CFTR (in the presence of forskolin plus IBMX), CFTR directly mediates both bicarbonate influx and efflux, contributing to pHi and pHe (Mastrocola et al., 1998). In NIH/3T3 and C127 cells, the effect of CFTR in pHe regulation was observed stimulating CFTR with forskolin (via cAMP and PKA) and ionomycin (via PKC activation) (Luckie et al., 2001). On the other hand, direct effects of CFTR in extracellular acidification were measured by time-resolved monitoring of metabolic activities in vitro (microphysiometry), in C127 cells (Luckie et al., 2014). In this work, the acidic extracellular pH of ΔF508 expressing cells was restored by using 10% glycerol for 24 h (glycerol restores ΔF508-CFTR to the cell membrane (Luckie et al., 2014)).

The CFTR chloride channel also participates in HCO_3^- secretion in pancreas (Hug et al., 2003), uterine endometrial cells (Wang et al., 2003), and duodenum (Spiegel et al., 2003). The permeability ratio HCO_3^-/Cl^- ranged from 0.10 – 0.27 in different cell types such as NIH/3T3 mouse fibroblast expressing recombinant wild-type CFTR (Poulsen et al., 1994), Chinese hamster ovary (CHO) cells (Linsdell et al., 1997), and Calu-3 lung adenocarcinoma epithelial cells (Illek et al., 1998; Illek et al., 1997). The CFTR channel also interacts with other bicarbonate transporters such as NBCn1 (SLC4A7), through its C-terminal domain, and with SLC26A6, via the STAS domain (Ko et al., 2002, 2004; Park et al., 2002; Shcheynikov et al., 2008; Wang et al., 2006). Furthermore, it also regulates the Cl^-/HCO_3^- anion exchanger (Lee et al., 1999). In addition, many studies have been done studying the role of CFTR in regulating luminal pH in small intestine (Illek et al., 1998; Jakab et al., 2012).

In airway epithelial cells, bicarbonate transport involves different transporters in both apical and basolateral membranes (Schultz, 2012). In human airway epithelial cells Calu-3, CFTR participates in Cl^- driven secretion of HCO_3- across the apical membrane (Shan et al., 2012). This secreted HCO_3^- appears to be generated by carbonic anhydrase located near the apical membrane (Shan et al., 2012). The same laboratory then reported that HCO_3^- secretion in Calu-3 cells is independent of pendrin (Huang et al., 2018), while other authors reported that CFTR acts together with pendrin channel (SLC26A4) to secrete HCO_3^-, in the same Calu-3 cells (Garnett et al., 2011). Thus, this issue is also a controversial matter. Other members of SLC26 family, such as SLC26A9 (Lohi et al., 2002), are also located in the apical surface of bronchiolar and alveolar epithelia, and participate in HCO_3-secretion. In fact, in human bronchial epithelial cells, there is a reciprocal relationship between SLC26A9 and CFTR, as SLC26A9 requires CFTR to function (Bertrand et al., 2009) but it also stimulates CFTR expression and activity (Avella et al., 2011).

8. CFTR and ASL pH

The CF pathophysiology is associated with persistent inflammation and infections in the respiratory epithelium (Nichols and Chmiel, 2015). These clinical manifestations result from dysfunctions in the airway epithelium, which takes part in the innate defense barrier, eliminating microbes and filtering the air. A vital component of the respiratory epithelium is the airway surface liquid (ASL) (Luan et al., 2014), which protects the epithelium from injuries and helps to fight against different insults. The ASL is composed of at least two layers, the mucus layer, and the periciliary liquid (PCL) interface. Its composition, volume, and clearance are crucial factors that equilibrate the respiratory epithelium homeostasis. In addition, the ASL contains several antimicrobial factors, including peptides and HCO_3^- (Knowles and Boucher, 2002).

Usually, the ASL pH varies from 6.85 to 7.65 (Fischer, 2012; Hunt et al., 2000). However, in many respiratory diseases, such as asthma (Hunt et al., 2000) or pulmonary tuberculosis (Ngamtrakulpanit et al., 2010), the pH homeostasis is altered and the ASL pH varies from ~4.5 to 8.5 (Fischer, 2012). In CF cells, Coakley at al. reported that the ASL pH appears to be acidic compared to normal cells (Coakley et al., 2003). Similar findings were observed in secretions from nasal submucosal glands (SMGs) (Song et al., 2006) or nasal ASL (Abou Alaiwa et al., 2014). The last work described a reduced ASL pH in CF neonates but not in children or adults, sustaining that the lack of differences perhaps occurs due to secondary manifestations of the disease. Similarly, a reduced pH was reported for exhaled breath condensates from CF patients (Ojoo et al., 2005; Tate et al., 2002). Moreover, in CF exacerbations (characterized by an upsurge of clinical symptoms and a decline in lung function), the decreased pH was even greater than in the CF stable disease (5.30 v 5.77) (Ojoo et al., 2005; Tate et al., 2002).

The ASL pH is affected by H^+ and HCO_3^- concentrations, and both CFTR and H^+ channels regulate the ASL pH in lung epithelium (Fischer, 2012). In addition, CFTR can interact with other channels to modulate ASL pH. It has been proposed that at an acidic ASL pH the voltage-gated hydrogen channel 1 (HVCN1) is closed and that HCO_3- is secreted through CFTR to alkalinize the ASL (Fischer, 2012). In CF airways, the dysfunctional CFTR does not secrete HCO_3^- to control the pH, and this might contribute to the acidic ASL pH (Fischer, 2012). On the other hand, there is also a reciprocal modulation between pHi and CFTR. Cytosolic pH modulates phosphorylation status of CFTR, changing its Cl- conductance (Reddy et al., 1998). The possible regulation of CFTR activity by pHe, on the other hand, has been controversial (Reddy et al., 1998; Sherry et al., 1994). The pHe also regulates other Cl- channels; for example, extracellular alkalinization stimulates calcium-activated chloride channels (CaCCs) in CF-cells (IB3-1) (Danko et al., 2011), which are an alternative way to increase Cl- transport in these cells (Schwiebert et al., 1998).

9. pH and immune function

An acidic extracellular pH may affect severely the immune function (Lardner, 2001). Indeed, pHe at inflammatory sites is often decreased (Lardner, 2001). Clinically, it is known the importance of HCO_3^- levels in serum, in different pathologies. Even in healthy people, low HCO_3^- levels are associated with high inflammatory markers (Farwell and Taylor, 2010). In vitro studies have shown that extracellular pH modulates the activity of different cells of the immune system (including dendritic cells) (Martinez et al., 2007), and also the complement system (Fishelson et al., 1987). Extracellular and intracellular pH regulation has been studied in neutrophils since it is crucial in modulating their microbicidal activity (Coakley et al., 2002). Exudative neutrophils showed impaired pHi regulation under extracellular acidosis (Hackam et al., 1996), and inhibition of the microbicidal activity of neutrophils was maximal at low pHe (Rotstein, 1993). Effects between acidic pHe and inflammation were also seen in alveolar macrophages activated with LPS, which showed decreased TNF secretion when the pHe values were diminished (Heming et al., 2001).

10. Extracellular pH influence in biofilms and bacterial strains in cystic fibrosis airways

As it was already mentioned, CF is characterized by recurrent lung infections and polymorphonuclear leukocytes infiltration (Ciofu et al., 2015). Although *P. aeruginosa, S. aureus, H. influenzae* and *B. cepacia* are the most common pathogens, other bacterial strains can also be present such as *Achromobacter xylosoxidans, Stenotrophomonas maltophilia* and nontuberculous *Mycobacteria* (Bhagirath et al., 2016). Some bacterial

strains form biofilms, which are constituted of one or more different species embedded in a matrix of polysaccharides (Ciofu et al., 2015). Biofilm formation increases antimicrobial tolerance compared to planktonic bacteria and facilitates evasion of the host immune system. Many of these biofilms are presented in CF patients lungs (Bjarnsholt et al., 2009; Hoiby et al., 2010; Starner et al., 2006), and affect severely their quality of life. Once these biofilms are established, they are difficult to eradicate. On the other hand, for several drugs the density-dependent growth inhibition is mediated by changes in pHe (Karslake et al., 2016).

In diseases different to CF, several pathogens (viruses, bacteria, fungi, etc.) take advantage of abnormal pH to increase their pathogenic infectivity and create mechanisms of adaptation to different tissues (Martinez-Rossi et al., 2017). Its role is important, for example, in *Candida albicans* infections (not considered a relevant pathogen in CF (Chmiel et al., 2014)), where acidic pH and low cAMP levels favor its growth as yeast (Danhof et al., 2016; Hollomon et al., 2016). Some viruses, like influenza virus, acidify the pHe when they multiplicate (Liu et al., 2016). In addition, many neutrophilic bacterial strains change their intracellular ATP concentration in response to extracellular pH variations, adapting their cellular bioenergetics to the new environment (Albert and Brown, 2015).

11. Recent findings regarding the ASL pH and the ASL clearance hypotheses

As mentioned above, in different in vitro and in vivo models the ASL pH was found acidic compared to their control counterparts, given support to the hypothesis that a low pHe might be a key factor in the susceptibility to infections. However, a recent report in CF children and in cultured primary cells sustains that the ASL pH does not change (Schultz et al., 2017). The authors compared CF vs. non-CF children with recurrent or chronic respiratory symptoms, between 1–6 years, using a novel fiber optic probe. They also used cultured primary CF and non-CF cells. The authors could not find differences in the ASL pH in vivo or in vitro, challenging the pHe hypothesis. They postulate that the lack of differences in the ASL pH between CF and non-CF cells might be explained by a paracellular acid/base shunt that compensates the lack of HCO_3^- transport through CFTR in CF cells.

On the other hand, as it was previously mentioned, using a newly developed synchrotron x-ray imaging analysis, Luan at al. showed that the introduction of bacteria, flagellin, or IL-1β into the lumen of intact isolated swine tracheas triggered CFTR-dependent ASL secretion by submucosal glands, an effect inhibited by CFTR(inh)-172 (Luan et al., 2014). Later, they found that this secretory response was impaired in *CFTR*(-/-) swine (Luan et al., 2017). These results suggest that the ASL secretion/volume would be reduced in CF patients, affecting the microbial clearance and leading to infections and inflammation, revitalizing the idea of a failure in the airway clearance (Button et al., 2012)(reviewed by (Quinton, 1989)), as the main factor influencing the susceptibility to infections. This IL-1β effect over the CFTR-mediated fluid secretion observed by Luan at al. (Luan et al., 2017) is in agreement with our early observation that IL-1β upregulates *CFTR* expression in colon T84 cells through NF-κB (Cafferata et al., 2001).

12. Factors that might contribute to the apparently contradictory results

Many factors might contribute to the different or contradictory results obtained. As Wine et al. pointed-out, differences may arise from the variety of methods used and from the degree in which the original pH is disturbed during measurements (Wine, 1999). They also sustained that, alternatively, perhaps each group is accurately measuring the pH but their cultures differ in the cells used or in the relative abundance of the different primary cells present in the culture. They concluded that there is not enough evidence to sustain that the cultures are comparable (Wine, 1999).

Another source of variability arises from the culture media used. Primary cultures of airway cells are made in the presence of FBS or serum substitutes, many of which have components of unknown composition (they often have up to 10 ng/ml EGF and a pituitary extract of unknown composition, among other uncertain components). FBS is plenty of growth factors that may affect differently each type of cells in the mixture (Gstraunthaler, 2003). EGF and the other factors used in serum substitutes might have unpredictable effects on the expression of CFTR, IL-1β, and many other genes (Ye and Lotan, 2008). The results could be more reproducible if well-defined serum-free media are used after cells reach confluence and differentiation, allowing them to acquire an unstimulated basal value. Of course, serum-free media have also pitfalls since they are deprived of important nutrients and in the long-term induce apoptosis (Barroso et al., 1997). Serum starvation also has a significant effect on the secretome composition (Eichelbaum et al., 2012). The ideal culture medium should perhaps include diluted calf serum (which has a reduced concentration of growth-factors compared to FBS), for which the optimal serum concentration may be found by comparing mRNA expression data (transcriptome) with the expression of the original in vivo tissue. A similar comparison has been already made with primary cultured cells from nasal tissues, but in the presence of an undefined media substitute without searching for an optimal amount of serum (a fixed amount of 2% Ultroser G was used) (Pezzulo et al., 2011). Another alternative, besides adding a known composition of nutrients, is to find a way to avoid apoptosis in serum-free media (Barroso et al., 1997). Of note, it is very important to consider that the presence of FBS or serum substitutes of unknown composition can mask autocrine loops (Chao et al., 1993), overstimulate/depress the expression of many genes (Ye and Lotan, 2008), induce oxidative stress (Chen et al., 2009), and interfere with measurements.

Schultz et al. have discussed other possible sources of controversies, including the lack of a CO_2 atmosphere in many in vitro measurements, the unknown and possible perturbing effects of changing the buffer in the apical region before measurements, and the lack of HCO_3^- in the media, masking the paracellular acid/base shunt (Schultz et al., 2017). In addition, they sustain that contradictory findings between young children and newborn pigs could be due to intrinsic pathophysiological differences among species. They also sustain that the human samples used represent a larger population that better represent genetic diversity compared to relatively uniform population in pigs or compared to previous studies with a small sample number and wider dispersion. One limitation was that they cannot rule-out mechanical effects affecting the glandular secretions, although this possibility was considered unlikely.

Nevertheless, at this stage we cannot rule-out that an intrinsic pH difference may exist between CF and non-CF cells, probably remaining latent due to compensation through a paracellular acid/based shunt, as it was suggested (Schultz et al., 2017). However, a system like this may be unstable, and any infection focus might trigger a stronger inflammation and a reduction in the surrounding pH, favoring infection spreading to other areas. Much work has to be done to better understand these apparently contradictory results.

13. Concluding remarks

Understanding the mechanisms involved in the establishment of an infection is crucial to develop drugs to counteract the microbiome involved in CF infections and related diseases. Here, we focused in one of the factors thought to be involved in the installation and persistence of infections in CF, which is the acidic pHe. To date, the link between CFTR and pH regulation relies on two aspects: the HCO_3^- transport mediated by the channel and the direct or indirect regulation of other transporters through CFTR. The CFTR failure in CF leads to a lower HCO_3^- transport that may result in an acidic pHe. It is thought that the acidic pHe in the lung milieu favors the establishment of infections in

CF. However, results from a previous work (McShane et al., 2003) and a recent one (Schultz et al., 2017), do not support the low ASL pH hypothesis as the factor that produces the lung susceptibility to infections, since changes in the ASL pH were not observed in these studies. The authors suggested that a tendency towards a reduced pHe will be balanced by a paracellular acid/base shunt, and that a reduced baseline ASL pH is unlikely to be an important pathobiological factor in early CF lung disease.

In conclusion, the issue is still highly controversial although the ASL dehydration hypothesis seems to be better supported by the evidence than the ASL pH hypothesis. On the other hand, recent findings using synchrotron x-ray imaging revitalized the earlier hypothesis of an affected ASL secretion, and, therefore, the airway clearance failure as the main factor determining the high susceptibility to infections in CF (Luan et al., 2017, 2014). Thus, the mucus hypersecretion, the low ASL pH, the increased ASL volume/dehydration or altered composition, and the ASL clearance hypotheses are all yet in debate. Most likely all these mechanisms, to a greater or lesser extent, contribute to the susceptibility to infections. Since Riordan cloned the CFTR in 1989 a considerable amount of knowledge has been acquired on the CF pathophysiology, but we have still a long way to go before the factors involved in the high susceptibility to infections in CF are clearly defined and understood.

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