Inflammation Research (2021) 70:633–649
https://doi.org/10.1007/s00011-021-01464-z

Inflammation Research

REVIEW

Hyperinflammation and airway surface liquid dehydration in cystic fibrosis: purinergic system as therapeutic target

Thiago Inácio Teixeira do Carmo[1] · Victor Emanuel Miranda Soares[1] · Jonatha Wruck[1] · Fernanda dos Anjos[1] · Débora Tavares de Resende e Silva[2] · Sarah Franco Vieira de Oliveira Maciel[2] · Margarete Dulce Bagatini[2]

Received: 24 February 2021 / Revised: 6 April 2021 / Accepted: 19 April 2021 / Published online: 27 April 2021

Abstract

Objective and design The exacerbate inflammatory response contributes to the progressive loss of lung function in cystic fibrosis (CF), a genetic disease that affects the osmotic balance of mucus and mucociliary clearance, resulting in a microenvironment that favors infection and inflammation. The purinergic system, an extracellular signaling pathway characterized by nucleotides, enzymes and receptors, may have a protective role in the disease, through its action in airway surface liquid (ASL) and anti-inflammatory response.

Materials and methods To make up this review, studies covering topics of CF, inflammation, ASL and purinergic system were selected from the main medical databases, such as Pubmed and ScienceDirect.

Conclusion We propose several ways to modulate the purinergic system as a potential therapy for CF, like inhibition of P2X7, activation of P2Y2, A2A and A2B receptors and blocking of adenosine deaminase. Among them, we postulate that the most suitable strategy is to block the action of adenosine deaminase, which culminates in the increase of Ado levels that presents anti-inflammatory actions and improves mucociliary clearance. Furthermore, it is possible to maintain the physiological levels of ATP to control the hydration of ASL. These therapies could correct the main mechanisms that contribute to the progression of CF.

Keywords Purinergic signaling · Airway surface liquid · Hyperinflammation · Cystic fibrosis

Introduction

Cystic fibrosis (CF) is an inherited recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which leads to abnormalities in the CFTR protein, a chloride (Cl^-) channel [1], with consequences mainly in the respiratory [2], gastrointestinal [3] and hepatobiliary tracts [4]. Allied to this, the dehydrated airway surface liquid (ASL) and an abnormal mucin secretion contribute to a persistent and progressive pulmonary inflammatory response [5].

CF is considered the most frequent autosomal recessive disease in the Caucasian population, with approximately 1/2500 live births [6]. More than 2000 CFTR mutations are already known to be associated with the disease [6–8] and are grouped into six classes according to their effect on the

Responsible Editor: John Di Battista.

✉ Margarete Dulce Bagatini
margaretebagatini@yahoo.com.br

Thiago Inácio Teixeira do Carmo
thiago.carmo@estudante.uffs.edu.br

Victor Emanuel Miranda Soares
victor.soares@estudante.uffs.edu.br

Jonatha Wruck
jonatha.wruck@estudante.uffs.edu.br

Fernanda dos Anjos
fernanda.anj@estudante.uffs.edu.br

Débora Tavares de Resende e Silva
debora.silva@uffs.edu.br

Sarah Franco Vieira de Oliveira Maciel
sarahfvo@gmail.com

[1] Medical School, Federal University of Fronteira Sul, Rodovia SC 484, Km 02, Fronteira Sul, Chapeco, SC 89815-899, Brazil

[2] Graduate Program in Biomedical Sciences, Federal University of Fronteira Sul, Rodovia SC 484, Km 02, Fronteira Sul, Chapeco, SC 89815-899, Brazil

protein. Variants classified as I, II, and III are associated with little or no CFTR function and are linked to a severe phenotype [7].

Class I mutations, also known as nonsense, frameshift or splice mutations, refer to the production of a truncated mRNA, causing the absence of the CFTR in the apical membrane. In class II mutations, both processing and maturation of the CFTR are compromised. These changes cause the protein to fail to fold properly and reach the apical membrane, being destroyed by the endoplasmic reticulum-associated pathway, reducing the number of active proteins. Regarding class III mutations, even if the protein reaches the membrane, the channel regulation is compromised, decreasing or blocking the passage of Cl^-. Class IV and V mutations are known to keep residual CFTR function, which generally preserves exocrine pancreatic function early in life. The conductivity of Cl^- is reduced in class IV mutations, which means that CFTR reaches the apical cell membrane, but there is a restriction in the ions flow. Towards class V mutations, the protein formation is functional, but due to alternative processing or reduced gene transcription, few proteins get to the membrane. Finally, class VI mutations cause the Cl^- flow to become unstable due to accelerated protein turnover on the cell surface [6, 7, 9, 10].

The pathophysiological manifestations differ according to the sensitivity of each organ to the functional deficit of the CFTR protein. The vas deferens are the ones that most need the proper functioning of the gene in question, since mRNA splicing is less efficient in the epithelia of the vas deferens, compared to the other affected organs [11–14]. For men, before birth, the vas deferens is blocked by viscous secretions and is thus reabsorbed, causing them to have congenital bilateral absence of the vas deferens in almost 95% of males with CF [14].

CFTR dysfunction causes pancreatic secretions to have a lower pH, lower secretory volume and hyperconcentration protein, leading to the obstruction of ducts and acini, causing progressive pancreatic damage, accompanied by inflammation, fibrotic processes and replacement by fatty tissue [15–18]. Furthermore, CFTR dysfunction in the sweat glands results in the loss of ions such as Cl^- and sodium (Na^+) in the sweat [19, 20]. Another clinical manifestation is the reduction of fluid secretion in the intestine, resulting in meconium ileus in the neonatal period, distal intestinal obstruction syndrome and constipation [21].

In the airways, ionic imbalance is caused by the absence or reduction in the Cl^- secretion mediated by CFTR, and by a continued activity of the epithelial sodium channel (ENaC), reducing the ionic gradients that induced the water movement into the airway lumen [22–26]. This results in a dehydrated surface, producing viscous, acidic and mucus-purulent secretions, which are difficult to eliminate. This thick mucus compresses the periciliary layer, affecting ciliary activity and mucociliary transport [27, 28]. Thus, decreased mucociliary clearance and altered ionic homeostasis allows colonization of the respiratory tract by bacteria [29] such as *Pseudomonas aeruginosa* [30–33], *Staphylococcus aureus* [34–36] and *Haemophilus influenzae* [37, 38]. These pathogens cause an inflammatory response, leading to chronic infection and possible loss of lung function (Fig. 1a) [29].

Therefore, in CF, there is a microenvironment conducive to recurrent infections, bacterial colonization and hyperinflammation [5, 39, 40]. CFTR deficiency is also associated with the deregulation of innate and acquired immunity. Thus, the inflammatory response of CF is marked by excessive numbers of neutrophils [5], eosinophils [41], macrophages with hyperinflammatory phenotypes [42] and pro-inflammatory mediators [5], which contribute to worsening lung function [43]. Thus, innovative treatments are sought to enable a better quality of life for patients, with purinergic signaling as attractive targets for new drugs against CF [44, 45], due to its important role in mediating inflammatory processes and the immune response [46].

The purinergic system includes purines and pyrimidines, enzymes (ectonucleotidases) that participate in the conversion of nucleotides and nucleosides, membrane transporters, and numerous subtypes of receptors (P1 and P2 families) responsible for the cellular response [47]. This purinergic signaling modulates the inflammatory response [48], once that activation of the P1 receptors, as A2A and A2B, play an anti-inflammatory and protective role in tissues [49, 50], and the activation of A1, A2B and A3 receptors promote inflammation and tissue damage [50, 51]. In addition to these, the P2 receptors, as P2X7 [52] and P2Y11 [53], are also involved in inflammatory and immune responses [52, 53]. Moreover, with a defective pathway of the Cl^-, the regulation of ASL in CF depends highly on extracellular purinergic signaling [22, 24]. Nucleotides, such as adenosine triphosphate (ATP), and nucleosides, such as adenosine (Ado), activate, respectively, P2Y2 and A2B receptors, which play an essential role in the generation of ionic gradients that regulate water flows and assist in ASL homeostasis (Fig. 1b) [24, 54–56].

Thus, this review addresses hyperinflammation in CF, its modulation by the purinergic system and its relationship with ASL dehydration. Finally, we propose therapeutics and future perspectives of the purinergic system for CF and its comorbidities.

ASL dehydration and hyperinflammation in CF

The pulmonary epithelium is covered by a layer recognized as ASL, which is crucial for maintaining homeostasis and preserving lung function. ASL is regulated by two

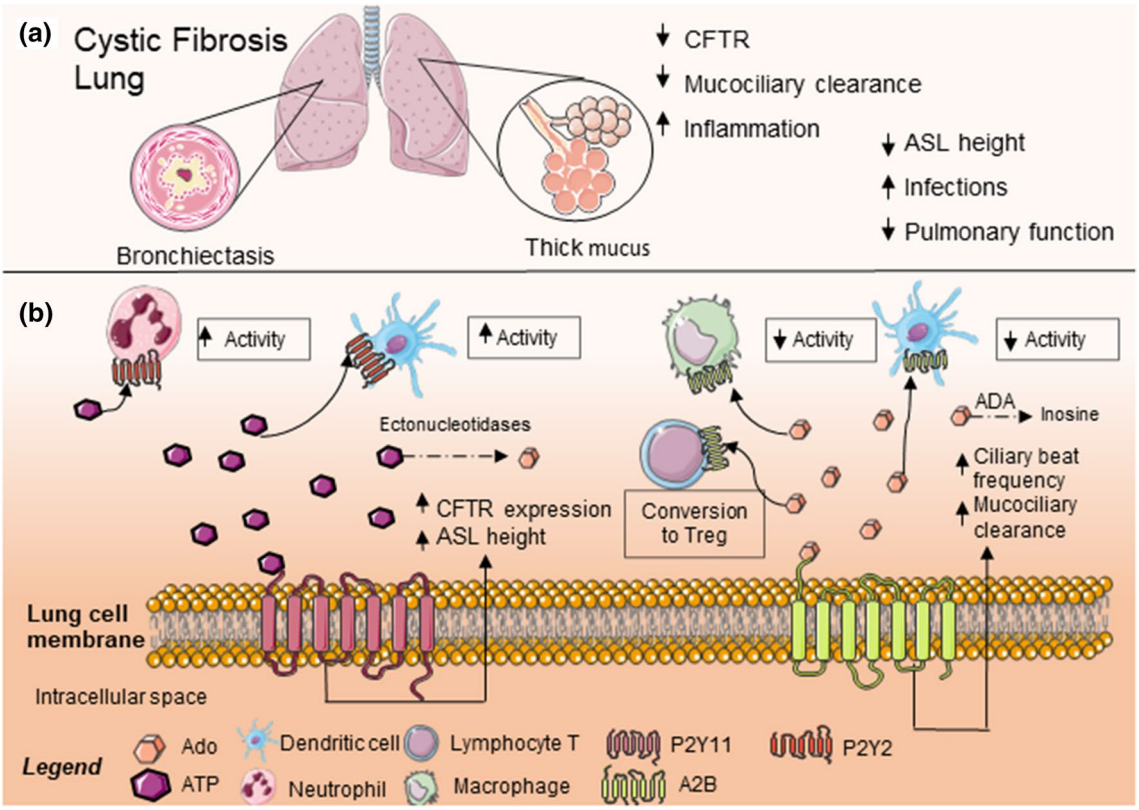

Fig. 1 Pathogenesis of CF and actions of the purinergic system that influence the pathophysiology of the disease. **a** The lungs of CF patients and their main molecular and structural changes. In the CF lung, reduced expression and/or functionality of the CFTR protein causes the mucus secreted by the goblet cells of the pulmonary epithelium to become thicker, which impairs the mucociliary clearance responsible for the elimination of pathogens, favoring infections and leading to chronic inflammation and bronchiectasis. In addition, the osmotic imbalance causes the ASL in CF to have a lower height compared to the airways of patients without the disease. These factors, in association, make the patient's pulmonary function considerably impaired and make up a large part of the pathophysiology of the disease. **b** The actions of the purinergic system on the pathophysiology of CF are represented. ATP interacts with the P2 receptors (like P2Y11 and P2Y2) in the respiratory epithelium, which increases CFTR expression and ASL height. In neutrophils, extracellular ATP and extracellular ADP, by means of P2Y6 receptors, act by increasing the release of pro-inflammatory cytokines, as well as their phagocytic activity, which ends up favoring inflammation. ATP can also interact with P2Y2 receptors in dendritic cells, increasing its pro-inflammatory activity. Following the cascade of the purinergic system, ectonucleotidases (CD39 and CD73) convert ATP and ADP into Ado. Ado, in turn, acts to suppress inflammation by stimulating A2A and A2B receptors in the cells of the immune system, which reduce the activity of dendritic cells and macrophages and direct T lymphocytes to the regulatory state (Treg), preventing their cytotoxic action and activation of other immune cells, and reducing tissue damage secondary to the inflammatory process. In addition, activation of Ado receptors in the pulmonary epithelium triggers a cascade of intracellular signaling that culminates in increasing the frequency of ciliary beats and consequently mucociliary clearance, which helps in the elimination of pathogens and, therefore, reduces infections and chronic inflammation. Finally, Ado is degraded to inosine, which ends the signaling cascade of the purinergic system

ion channels: ENaC and CFTR [57]. A new model was described by Roesch et al. [5], called the "two-gel model", according to which states that the ASL is composed of the periciliary gel and the periciliary layer. The periciliary gel is dense, non-uniform and is compacted towards the epithelial cell surface. The periciliary layer, on the other hand, is composed of mucins, (MUC1, MUC4, MUC16 and MUC20) that increase the ability to attract water to the periciliary layer to hydrate it and for the mucus to slide over the gel. However, in pathological conditions such as CF, water is removed from the periciliary gel, leading to mucus hyperconcentration, decreased mucociliary clearance and mucus adhesion to the airway walls.

The two layers work on the gel-on-brush model, in which the mucins and mucopolysaccharides from the periciliary layer form a network of brush-shaped polymers on the epithelial surface. This system creates a semi-permeable gradient mesh and acts as a size exclusion barrier for infiltrating entities [58]. Another important mechanism is the removal of pathogens that have been inhaled through the unidirectional movement of the mucus layer, and the cilia that project from the epithelium of the airways to the aqueous periciliary

layer close to the epithelium, promoting elimination towards the epiglottis [59]. The periciliary brush also contributes to the regulation of ASL layer hydration by facilitating water distribution between the two layers [58].

To regulate the height of the ASL, a combined action of several ion channels on the apical surface of the airway epithelium is necessary. CFTR, calcium-activated chloride channels (CaCCs) and ENaC are responsible for regulating Cl^- and Na^+ transport. These channels on the apical surface regulate ASL hydration by secretion of Cl^- across CFTR and CaCCs, and epithelial Na^+ absorption by ENaC [60].

In that perspective, all mutations in CFTR have a negative impact on decreased Cl^- secretion and consequently on increased Na^+ reabsorption in the cell space [61]. Because of this, CF patients generally have thicker mucus secretions in the epithelial linings and more viscous secretions from exocrine tissues [62]. Therefore, in the pulmonary context, ASL dehydration is related to severe consequences such as bronchiectasis, infection and persistent airway inflammation [63].

ASL dehydration is the result of an ionic imbalance caused by the absence or abnormalities in the Cl^- secretion mediated by CFTR and by a continued ENaC activity, generating a reduction in the ionic gradients that generate water flow [23–25, 64]. These changes are intrinsically related to hyperinflammation due to airway obstruction and promote a favorable site for infection and bacterial colonization [5, 39, 40].

In CF patients, airway infection is accompanied by exaggerated inflammation [40], because the normal functions of airway epithelial cells and the modulation of the inflammatory and immunological responses are impaired [65]. In addition, ASL dehydration favors the colonization of opportunistic bacteria, as the airways are the gateway and one of the first places of contact with pathogens [39], such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* [65] and *Aspergillus fumigatus* [66].

CFTR deficiency is also associated with cell membrane lipid abnormalities and dysregulation of innate and acquired immunity [67]. Furthermore, the exorbitant number of immune cells, such as neutrophils, and increased concentrations of pro-inflammatory mediators, such as tumor necrosis factor alpha (TNF-α), interleukins (IL-1, IL-6, IL-8, IL-17, IL-33), granulocyte–macrophage colony-stimulating factor (GM-CSF or G-CSF) and high-mobility group protein 1 (HMGB-1) [5] during the inflammatory response in situations of pathogen invasion, contributes to the progressive loss of lung function, worsen airway obstruction, and causes structural damage to the airway wall architecture [68].

The immune response starts with toll-like receptors (TLRs) [69] expressed in the pulmonary epithelium that recognize the lipopolysaccharides (LPS) of the membrane of pathogens. After recognition, pro-inflammatory cytokines are produced by activating intracellular molecules such as kinases associated with the IL-1 receptor (IRAKs) [70]. Thus, there is a predominant inflammatory influx of neutrophils [71], which are chemo-attractive to immune cells responsible for maintaining and amplifying the excessive inflammatory response and, therefore, can be a good biomarker for pulmonary exacerbation of CF [72]. Neutrophils have antimicrobial functions through the generation of reactive oxygen species (ROS), secretion of antimicrobial peptides, phagocytosis of microorganisms and trapping bacteria in extracellular neutrophil traps (NETs) [73]. However, in CF, these cells are present in excess, causing the normal defense functions of the host to become pathological [5].

Koller et al. [74] claim that eosinophils can be more activated in the airways of CF patients, and contribute to the exaggerated inflammatory response. In this study, high serum levels of eosinophil cationic protein (ECP), an eosinophil activation marker, were detected in CF patients. Similar to the increase in eosinophils in CF patients, basophils can also be more activated, but are not more abundant [75].

Macrophages are another important immune cell for building adequate host defense. During the acute response, chemokines and cytokines are released, which further promote the attraction of neutrophils and monocytes [42]. When monocytes reach the lungs or airways, they develop into macrophages, but hypoxia in the upper lung lobes in patients with CF, where oxygen tension is lower, causes the alveolar macrophages to produce in greater quantities TNF-α and IL-8 [5]. In addition, these cells demonstrate a hyperinflammatory phenotype, because they cannot migrate from classes and present impaired efferocytosis [42].

Under normal conditions, monocytes, after being recruited into the lungs, are differentiated into macrophages (M0), which can polarize to type 1 (M1) with pro-inflammatory properties, or type 2 (M2) with anti-inflammatory properties. Moreover, these cells have a property called plasticity, which allows them to migrate between M1 and M2 to regulate immune responses [42]. In this context, M0 macrophages, when exposed to interferon-Gamma (IFN-γ), GM-CSF or LPS, become M1, which in turn expresses CD80 on the cell membrane, causing high amounts of ROS and secretion of pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6, IL-12, IL-23, IFN-γ, C–C motif chemokine ligand 2 (CCL2) and regulated on activation normal T cell expressed and secreted (RANTES). In addition, M1 macrophages have antimicrobial and phagocytic activities and secrete little IL-10. On the other hand, polarization in M2 macrophages occurs due to exposure to IL-4, IL-13, IL-10 or macrophage colony-stimulating factor (M-CSF). M2 macrophages are cells associated with the healing and tissue repair process, they inhibit the polarization of M1 macrophages, are responsible for the secretion of IL-4 and IL-13, have endocytotic functions and express scavenger

and mannose receptors, IL-1 receptor antagonist, CD209, arginase-1 and interleukin-13 receptor alpha 1 (IL-13Rα1). Phagocytosis by macrophages is also impaired due to the low expression of cluster of differentiation molecule 11B (CD11b), a receptor for opsonic phagocytosis [5].

Regarding the mechanisms that promote an excessive inflammatory response in CF, T cells also play an important role. This is because the Treg cells are reduced, which suppress the responses of T-helper type 2 cells (Th2), which release IL-4 and IL-13, and of Th17 cells, which release IL-8 and IL-17, promoting the flow of neutrophils that amplify the inflammatory response by secreting chemokines C-CCCL2 and CCL20, which recruit more Th17 cells [5]. B lymphocytes are also altered in CF and contribute to inflammation in the airways, probably playing the role of antigen presenting cells [75].

In addition, repeated infections and pulmonary colonization contribute to hyperinflammation and extensive inflammatory changes in the airways, resulting in sequelae, bronchiectasis and progressive airflow obstruction, which can be life limiting [76]. Therefore, it is necessary to study new therapeutic potentials to regulate ASL and control the inflammatory process resulting from CF.

Purinergic system role in CF

The term "purinergic" was proposed in 1972, after professor Geoffrey Burnstock found ATP neurotransmission in non-cholinergic and non-adrenergic nerves [77]. Initially, there was resistance to this concept, since ATP was such a simplistic and ubiquitous compound in the body, so it would be unlikely to be used as an extracellular messenger [78]. However, almost 50 years after this first discovery, the purinergic system is already consolidated in numerous pathophysiological processes, such as inflammation [79], tromboregulation [80, 81], pain conditions [47], tumor growth [82–84], neurodegeneration [83, 85], psychiatric disorders [86] and others. Thus, modulation of the purinergic system has been an increasingly promising area for the development of therapeutic strategies [87, 88] for diseases, such as inflammatory ones like CF [89, 90].

The purinergic system is composed by enzymes (ectonucleotidases) that participate in the conversion of nucleotides and nucleosides of purines and pyrimidines, membrane transporters and various subtypes of receptors responsible for the cellular response [47]. The four subtypes of P1 receptors (A1, A2A, A2B and A3) are activated by Ado. P2 receptors are activated by purines and some subtypes also by pyrimidines. The P2X receptor comprises seven subtypes (P2X1-P2X7) that function as ion channels. In addition, there are eight receptor subtypes coupled to G protein, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 [91]. Ectonucleotidases such as ectonucleoside triphosphate diphosphohydrolase-1 (CD39), which converts ATP/adenosine diphosphate (ADP) to adenosine monophosphate (AMP), and ecto-5′-nucleotidase (CD73), which converts AMP to Ado, play essential roles in modulating purinergic signals [92]. Following this pathway, adenosine deaminase (ADA), an enzyme present both inside the cell and on the cell surface, degrades adenosine to inosine or 2′deoxyadenosine to 2′deoxyinosine (Fig. 2) [93].

Ado and ATP/ADP are extracellular mediators that act on G protein-coupled receptors. Ado is a central molecule in CF, directly and indirectly regulating CFTR, the volume of ASL and inflammation (Fig. 3) [79]. In addition, it controls the ionic transport in the epithelium of numerous tissues, such as the gastrointestinal, respiratory and renal tracts, due to its ability to activate all four P1 receptor subtypes. Meanwhile, ATP/ADP is able to activate P2 receptors or to be readily converted to ADP and AMP. AMP can still be converted to Ado. The activation of P2 receptors has therapeutic potential in inflammatory diseases, such as CF, because it acts by modulating immune responses [56]. In addition, some of these receptors can modulate the ionic flow of Cl^- and Na^+ [94], improving mucociliary clearance [95] and even modulating CFTR function [96]. Table 1 shows the main actions of activation and blocking of the purinergic system receptors applied to CF pathophysiology.

The inflammatory response in CF causes many problems to patient health conditions, such as structural airway damage, airway obstruction, deterioration of host defenses and progressive loss of lung function [5]. In this context, ATP acts on a variety of inflammatory cells as a molecule associated with danger, signaling inflammatory responses through P2 receptors [97]. It is also related to chemotaxis [98] and the production of inflammatory cytokines such as IL-1, IL-6, among others, as shown in Table 1 [52, 99].

Junger [100] indicates that P2X7 receptor is mostly expressed in inflammatory cells, which means that this receptor seems to be present in ATP-induced inflammation [101]. However, even presenting this inflammatory response induced by ATP, P2X7 receptor has lower affinity to ATP in comparison to other P2 receptors [102]. When stimulated, P2X7 receptor provokes inflammasome activation [103], the release of cytokines and chemokines and the differentiation of T-helper lymphocytes to cytotoxic T lymphocytes [52]. These inflammatory effects result from high levels of extracellular ATP, as can be observed in the first stages of inflammatory status [104].

The activation of P2X7 is correlated with the release of TNF-α from dendritic cells, which is important for the activation of the immune system in general and, consequently, for the elimination of pathogens [105]. However, besides stimulating the release of cytokines commonly involved in CF-related inflammation pathogenesis, such as TNF-α,

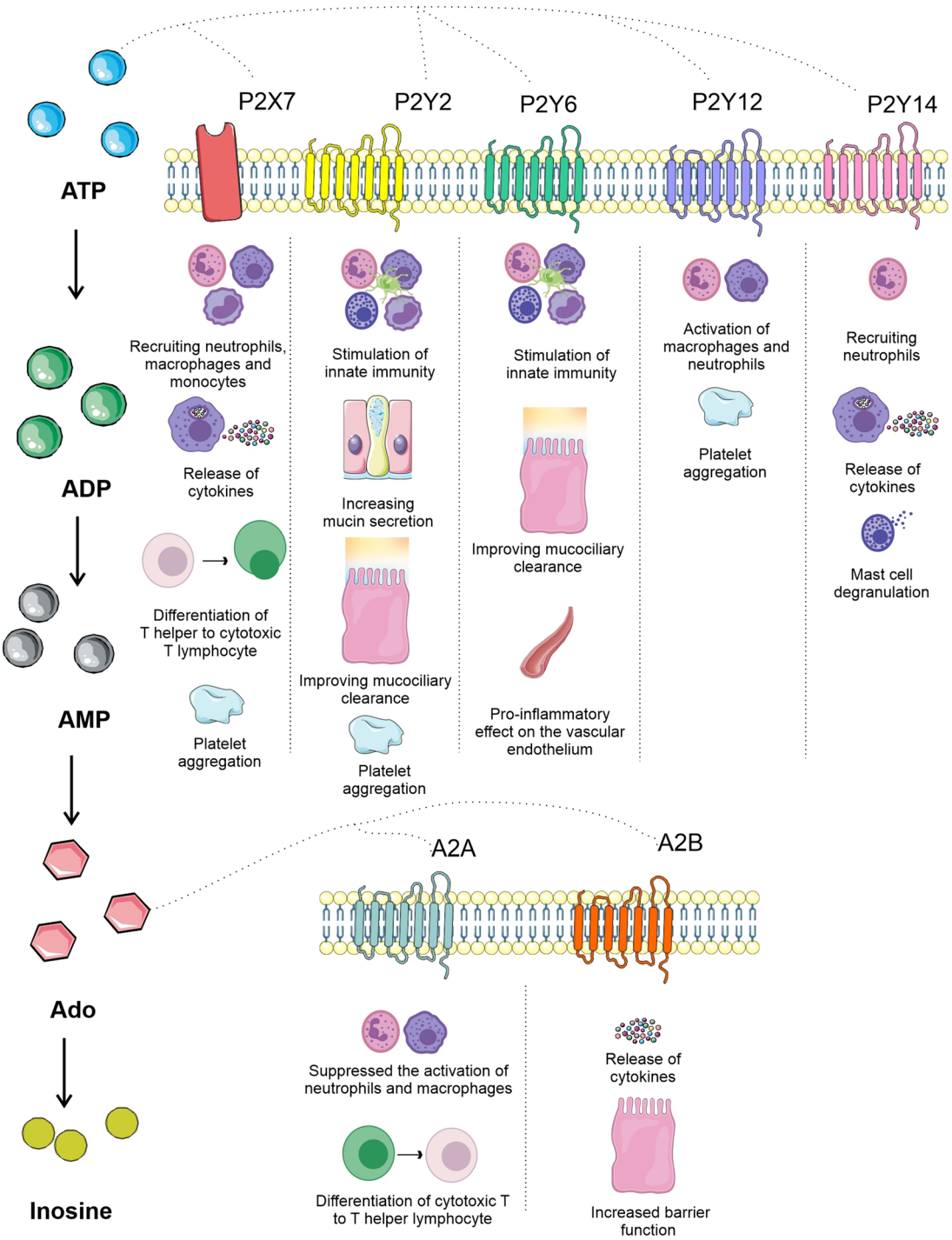

Fig. 2 The role of the purinergic system in CF begins with the action of ectonucleotidases in the cascade that convert adenosine triphosphate (ATP) to adenosine diphosphate (ADP) into adenosine monophosphate (AMP), AMP to adenosine (Ado) and finally, Ado in inosine. In this figure, the main receptors activated by ATP and Ado are represented. ATP basically acts on a variety of inflammatory cells as a molecule associated with danger, release of cytokines and signaling inflammatory responses. On the other hand, Ado controls inflammation by converting T lymphocytes to the regulatory state (Treg), suppresses the action of macrophages and neutrophils and increases barrier function

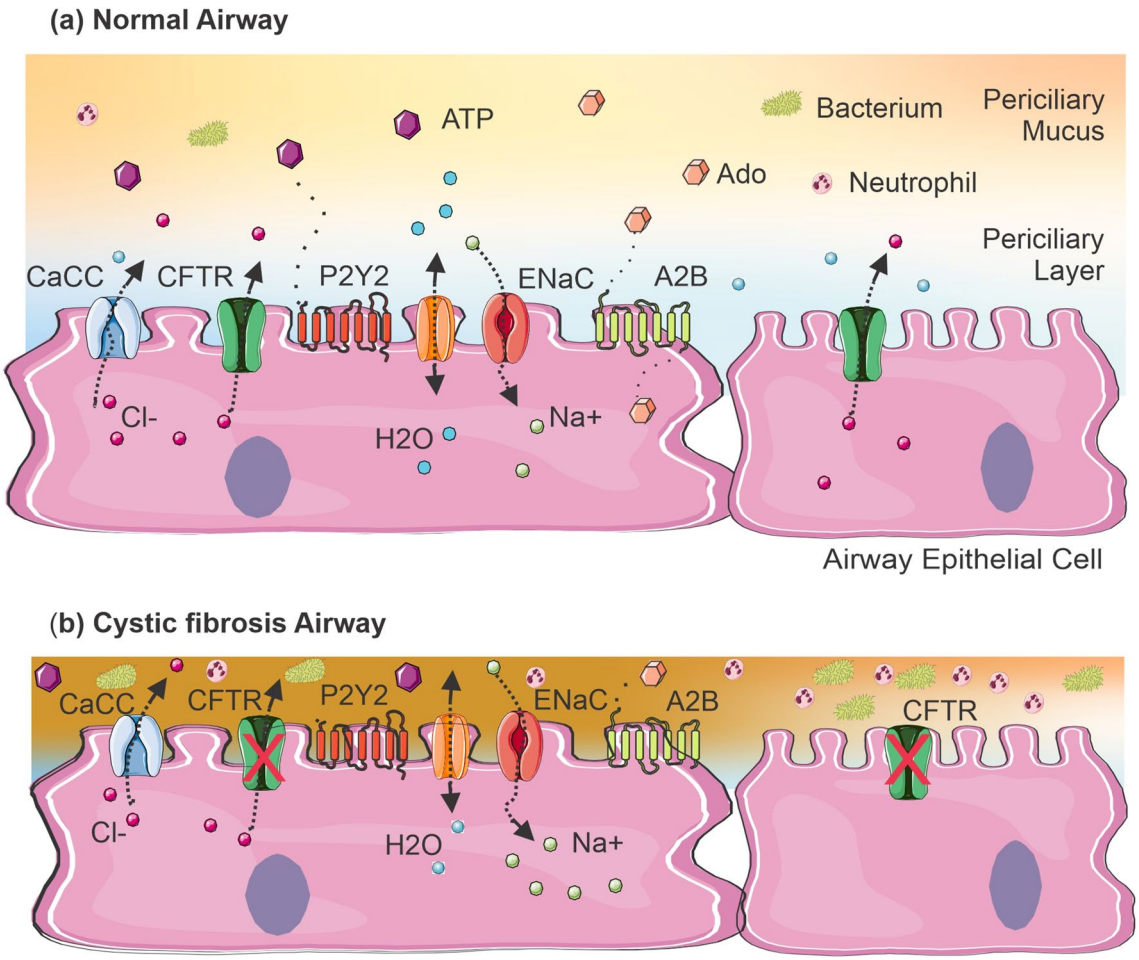

Fig. 3 Regulation of ASL in normal and CF airways, and its interaction with the purinergic system. **a** Extracellular ATP activates P2Y2, which stops Na^+ absorption and Cl^- secretion by CFTR and CaCC. Ado signals via A2B, which generates cAMP that stimulates Cl^- secretion via CFTR. In a CF-mutated cell (**b**), the influx of Ca^{+2} and the impaired or null efflux of Cl^- by CFTR promotes the entry of water into the cell, ASL dehydration, and inflammation

IL-1β, IL-6, IL-8, IL-17, the P2X7 receptor also inhibits the release of anti-inflammatory cytokines, such as IL-10 and transforming growth factor beta (TGF-β), which contributes to the durability and exacerbation of an inflammatory state, increasing secondary tissue damage [106]. In the airways, the binding of ATP to P2X7, mainly in the vascular endothelium, also promotes local inflammation, with recruitment of leukocytes, release of pro-inflammatory cytokines and stimulation of platelet aggregation [79].

Although the pathophysiology of chronic lung diseases are diverse, a common feature among these conditions, and very present in CF, is the excessive recruitment and unregulated activation of effector cells, including neutrophils, eosinophils, macrophages, airway epithelial cells, fibroblasts and myofibroblasts, leading to the release of more mediators which, together, potentiate lung inflammation and tissue remodeling [79]. In this sense, as seen the role of P2X7 activation in the exacerbation of inflammatory conditions, its blocking could be beneficial for the improvement of pulmonary function in CF patients, since it has been effective in reducing inflammatory cells and secretion of pro-inflammatory cytokines, as well as increasing IL-10 secretion in models of chronic obstructive pulmonary disease [107]. This inhibition of the P2X7 receptor can be tested for CF through the use of already existing receptor antagonists, such as JNJ-54175446, which has promising clinical studies to control inflammatory diseases of the central nervous system [108, 109]. In addition, the fact that P2X7 knockout rats showed considerably reduced fibroblast disposition and inflammatory damage in contact with silica compared to control rats [106], demonstrates, once again, the possible beneficial effect of blocking these receptors to control chronic inflammatory diseases such as CF.

Binding extracellular nucleotides to P2Y receptors promotes a cascade of signaling that triggers a series of cellular effects that lead mainly to the stimulation of innate immunity

Table 1 Main receptors of the purinergic system and their actions in CF

Receptors	Effect of activation on CF	Effect of blockade on CF	References
P2X7	Increased release of pro inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IL-17, and reduced release of anti-inflammatory cytokines such as IL-10 and TGF-β; increased immune response with reduced bacterial infections	Reduction of fibroblast deposition; reduction of the inflammatory process	[79, 105–107]
P2Y2	Infection control; stimulation of dendritic cell activity during chronic lung diseases; improved mucociliary clearance and increased mucin secretion in the airways; promotion of exaggerated inflammatory states	Inflammation reduction; mucociliary clearance reduction; increased infections	[56, 79, 101, 166]
P2Y6	Increased inflammation, especially in the vascular endothelium; increased pulmonary fibrosis; improvement of mucociliary clearance; innate defenses activation against infections	Better outcomes in inflammatory diseases; reduction of inflammation and pulmonary fibrosis	[78, 79, 112–114]
P2Y11	Increased ATP secretion mediated by LXA4, which shows a reduction in inflammatory mediators without increasing the infectious load; increased expression of CFTR protein and restoration of ion transport in the bronchial epithelium, also through joint work with LXA4; increased height of the airway superficial liquid; delay of *Pseudomonas aeruginosa* infection	Significantly reduces the height of LXA4-induced surface airway fluid	[53, 119, 164, 165]
P2Y12	Related to asthma attacks; activation of macrophages and, especially, of neutrophils	Reduction in levels of inflammatory mediators associated with platelets such as soluble P-selectin and CD40-L, as well as systemic inflammatory biomarkers such as TNF-α and C-reactive protein; reduction in lung injury	[101, 116–118]
A2A	Conversion of cytotoxic T lymphocytes to the Treg state; Suppression of macrophage and lymphocyte activity; improvement of mucociliary clearance; increasing the frequency of airway ciliary beats	Increased pro-inflammatory cytokines; Increased immune cell response against pathogens	[54, 55, 134, 167]
A2B	Reduced levels of pro-inflammatory cytokines, such as TNF-α and IL-6 in bronchoalveolar lavage; increased mucus hydration and ciliary beat frequency; improvement of mucociliary clearance	Increasing macrophage phagocytic capacity; improvement in the control of bacterial infection; increased secretion of IL-6 and TNF-α	[54, 55, 79, 135, 166, 167]

and platelet aggregation [110]. Acting on caliciform cells provides an important route for regulating mucin secretion [78]. In this sense, P2Y2 receptor is essential for infection control, once it activates innate immunity [101] and, more specifically, during chronic lung diseases it acts by stimulating dendritic cells activity [79]. In addition, P2Y2 activation has been effective in improving mucociliary clearance and increasing mucin secretion in the airways, improving initial defenses against exogenous pathogens [101].

On the other hand, the study of P2Y2 agonists revealed a dual role of the receptor, a positive effect when acting in infection control [101], but also a negative effect when promoting exaggerated inflammatory states [56]. In this sense, although the use of P2Y2 receptor agonists seems promising for the treatment of CF, their pro-inflammatory role suppresses their beneficial effect, which is a possible explanation for the failure of 2′-desoxycytidine(5′)tetraphospho(5′) uridine (Denufusol®) in clinical studies, for example [111].

Unlike other P2Y receptors, which are rather activated by ATP, the P2Y6 receptor has uridine diphosphate (UDP) as the main endogenous binding agent and, to a lesser extent, uridine triphosphate (UTP). This receptor is coupled to a Gq protein and induces lipid signaling of inositol through phospholipase C [110], with a pronounced pro-inflammatory effect on the vascular endothelium [79]. In CF patients, the release of uridine nucleotides such as UTP, UDP-glucose and UDP are increased [101]. In addition, P2Y6 receptor is more expressed in respiratory epithelium cells of patients with pulmonary fibrosis than in healthy lungs [112], and pharmacological inhibition or genetic exclusion of the receptor in murine models of intestinal inflammation was associated with better outcomes of the disease [113]. Together, these results indicate that activation of this receptor can be an important mediator of inflammation and fibrosis in CF and, therefore, its blocking has therapeutic potential for the disease.

On the other hand, the role of P2Y6 receptor in activating innate immune response and improving mucociliary clearance was reported, and this is fundamental for the prevention and control of infections that are part of the CF physiopathology [114]. However, the pro-inflammatory effect of the receptor makes it difficult to use P2Y6 agonists to improve mucociliary clearance in CF patients [78]. These results indicate that although P2Y6 activation is important for innate defenses against infections, inadequate signaling through this receptor can lead to exaggerated immune response in chronic inflammatory disorders such as CF.

Lastly, P2Y12 receptor has a signal transduction mechanism that inhibits adenylate cyclase, which reduces the intracellular concentration of cyclic adenosine monophosphate (cAMP) [115]. It is expressed mainly in platelets and, therefore, its main function is to regulate hemostasis [101]. The action of P2Y12 on platelets favors coagulation and, thus, inhibitors of this receptor, as clopidogrel and ticagrelor, have long been used as anticoagulants [116]. However, since platelets are important liberators of inflammatory mediators, their action in inflammatory diseases have also been studied [101].

Patients using clopidogrel and ticagrelor showed a transitory reduction in levels of inflammatory mediators associated with platelets, such as soluble P-selectin (protein responsible for leukocyte adhesion to the vascular endothelium in the early cascade of events that lead to the inflammation process) and CD40-L (protein expressed primarily in activated T cells that binds to CD40 in antigen presenting cells, which leads to many effects depending on the type of target cell), as well as in systemic inflammatory biomarkers, such as TNF-α and C-reactive protein [117].

In addition, it was observed that ticagrelor is a more potent P2Y12 inhibitor than clopidogrel, and can improve clinical outcomes of lung infections [117]. Another study showed that mice with severe lung infections treated with clopidogrel, or even in the absence of P2Y12 receptor, showed considerable reduction in lung injury and, therefore, better clinical and laboratory results. Besides that, since the receptors involved in platelet responses to ADP are P2Y1 and P2Y12, P2Y1 has also been tested. No difference was observed in null mice for this receptor during sepsis, indicating that P2Y12 receptor is responsible for the effects of reducing lung injury and improving clinical outcomes [118].

In the pulmonary context, it was observed that P2Y12 activation was related to other exaggerated inflammation conditions similar to CF, such as asthma attacks. Also, an important role of purinergic system receptors, mainly of the P2 type, in the activation of macrophages and, especially, neutrophils, was reported, the latter being the main cell type constituting the CF inflammatory environment [101, 119].

Recently, a study suggested that UDP glucose released into the airways acts as a local mediator of inflammation in CF, recruiting neutrophils, through the stimulation of P2Y14 receptor [120]. In addition, activation of this receptor is related to mast cell degranulation [121] and release of pro-inflammatory cytokines [122]. Thus, there are studies on the chemical and structural basis of P2Y14 antagonists, as of triazole derivatives [123], which can potentially result in the development of clinically useful drugs to treat inflammation in CF [124]. In its turn, Ado presents opposite effect to ATP, causing reduction of inflammation status by inhibition of endothelial adhesion, reduction of superoxide anion production by neutrophils and decreasing release of pro-inflammatory cytokines [125–127].

There are evidences of worsening mucosal inflammation in mice with genetic deletion of enzymes responsible for Ado metabolism, such as CD39 and CD73, that causes reduction of extracellular Ado levels and signaling, even with high levels of ATP and ADP, which evidence the Ado

role in the anti-inflammatory response [128–130]. In this sense, Fredholm [131] indicates that Ado plays an anti-inflammatory and tissue protective action by activation of A2A and A2B receptors [49, 50, 131], or pro-inflammatory and tissue damage by activation of A1, A2B and A3 receptors [50, 51].

Ado receptors are specific for each target cell and have anti-inflammatory actions and stimulate Cl^- secretion, important properties for the control of CF [79, 132]. The A2A receptor is highly expressed in immune system cells such as neutrophils and lymphocytes. The binding of Ado to A2A receptors of T lymphocytes leads to their conversion to the regulatory state (Treg), suppressing inflammation [79]. It is worth mentioning that GW328267C, an A2A receptor agonist, has been shown to improve lung function in acute injuries [133]. In addition, the action of macrophages and neutrophils is also suppressed by the receptor and, therefore, the use of A2A agonists in inflammatory diseases such as CF is promising [133, 134].

A2B receptor is very expressed in pulmonary endothelium cells, and when it binds to Ado, shows increased barrier function and reduced inflammation [79]. A2B inhibition resulted in reduced levels of pro-inflammatory cytokines, such as TNF-α and IL-6 in bronchoalveolar lavage, signaling the effect of the receptor to suppress inflammation in the lungs. On the other hand, A2B inhibition results in improved bacterial infection control by increasing macrophage phagocytic capacity. After direct stimulation of inflammation by bacterial LPS in vivo, septic macrophages from knockout mice for A2B receptors had increased secretion of IL-6 and TNF-α compared to wild-type mice [135]. An A2B receptor agonist, BAY60-6583, showed improvement in acute lung injury [136–138] by inhibiting epithelial cell apoptosis [138], increasing alveolar fluid clearance [136], and attenuating inflammation [136, 137]. Thus, treatment with A2B agonists, such as BAY60-6583, can also be beneficial for CF.

In short, ATP levels are higher in early inflammatory response, increase inflammatory status, and tend to get low by the action of CD39 and CD73, working later as an anti-inflammatory molecule. Ado tends to get higher by the action of CD39 and CD73, and its action tends to inhibit inflammatory response [139]. Taken together, these results indicate that changing the balance of purinergic signaling from P2 to P1 receptors may be interesting for the treatment of inflammatory diseases, such as CF. This can be done by increasing the activity of CD39, which converts ATP/ADP to AMP; and CD73, which converts AMP to Ado. Thus, the concentration of ATP, which activated P2 receptors, causing a pro-inflammatory state, is reduced, while the concentration of Ado, which activates P1 receptors of mostly anti-inflammatory activity, will increase [140]. However, to date, there are no compounds proven to increase ectonucleotidase activity. Therefore, it is important that future research seeks to analyze compounds with such properties so that the therapeutic strategy in question is tested in a practical way.

Another way to increase Ado concentrations and obtain its anti-inflammatory effects would be by inhibiting ADA. In that case, there are many potent ADA inhibitors that could be tested [93], including drugs, such as coformycin analogues [141] and non-steroid anti-inflammatory drugs [142], and natural phenolic compounds, such as curcumin [143]. We postulate that the advantage of ADA inhibition over CD39 activation as a possible therapy for CF is that the former maintains the ATP levels necessary for ASL homeostasis, which will be discussed later. To date, no studies have been carried out to test ADA inhibitors specifically for CF; however, given the above, it is clear that they have great potential for disease control and therefore deserve to be tested.

Role of purinergic signaling in ASL regulation

The inability to maintain mucociliary clearance in airway epithelia promotes lung diseases, such as CF [22], in which water is removed, causing mucus adhesion to the airway walls [5]. Paracellular and transcellular movement of ions and water are responsible for the hydration mechanism of the mucus layer, as well as by the volume of periciliary liquid [61]. The main ions responsible for ASL homeostasis are Cl^- and Na^+, and their concentration in ASL are between ~ 100 and 130 nM, followed by potassium (K^+; ~ 20 nM) and bicarbonate (HCO3-; ~ 10 nM). The airway epithelial ion transport is determined by the electrochemical gradient [144, 145].

In CF, CFTR, the main Cl^- channel responsible for the apical secretion of this ion, is not functional, however, CaCCs such as anoctamin-1 (ANO1), also known as transmembrane member 16A (TMEM16A), and the solute carrier family 26 member A9 (SLC26A9), also work to Cl^- secretion, while the apical big potassium channel (BK), which is a Ca^{2+}-activated and voltage-dependent potassium channel, hyperpolarize the apical membrane, working as a counterion to increase the force to Cl^- secretion [144, 145].

To understand the ASL regulation, Button et al. [146] have tested if ATP and Ado play the role of ASL height regulation and hydration in steady state, by nebulization of a vehicle containing apyrase and ADA to degrade extracellular ATP and Ado, respectively, which led to the completely ASL dehydration and cilia compression, attesting the role of those molecules in ASL regulation. Table 2 shows in a summarized way the therapeutic strategies and molecules that we believe should be tested for CF, because of their considerable therapeutic potential.

This evidence is reinforced by Tarran [147], whose research suggests that the absence of ATP and Ado

Table 2 Possible therapeutic strategies and molecules that should be tested for CF

Physiological action predicted	Receptor or enzymes envolved	Therapeutic modulation	Compounds	References
Reduction of inflammatory cells; Reduction in secretion of pro-inflammatory cytokines; Increase of IL-10 secretion; Reduction in fibroblasts deposition and inflammatory tissue damage	P2X7 receptor; ATP	Block	JNJ-54175446	[106–109]
Activation of innate immune response; Stimulation of dendritic cells activity; Improvement of mucocilary clearance; Increase in mucin secretion	P2Y2 receptor; ATP	Activation	2′-desoxycytidine(5′)tetraphospho(5′)uridine	[79, 101]
Reduction in coagulation; Decrease of the levels of pro-inflammatory mediators; Anti-inflammatory action	P2Y12 receptor; ATP	Block	Clopidogrel; Ticagrelor	[101, 116, 117, 119]
Decrease in local inflammation; Reduction of Mast cell degranulation; Reduced release of pro-inflammatory cytokines	P2Y14 receptor; UDP	Block	Triazol derivatives	[120, 122, 123]
Reduction of inflammatory status; Reduction of superoxide anion production; Reduce in pro-inflammatory cytokines release	Ado; CD39; CD73; A2A receptor; A2B receptor; ADA	Block of the Ado metabolism by ADA; Activation of A2A and A2B receptor; Increase in CD39 and CD73 levels	Coformycin analogues; Non-steroid anti-inflammatory drugs; Curcumin	[93, 125–130, 141–143]
Reduce of inflammation; Suppression of macrophages and neutrophils action	A2A receptor; Ado	Activation	GW328267C	[79, 133, 134]
Increase in barrier function of endothelium cells; Reduce of inflammation; Reduce of pro-inflammatory cytokines levels	A2B receptor; Ado	Activation	BAY606583	[79, 135–138]
Inhibition of Na^+ absorption by ENaC; Increase in Cl^- secretion by CaCC and ANO1/TMEM16A; Increase in K^+ efflux by BKs and CaKCs; Increase ASL height	P2Y2 receptor; ATP; CD39; CD73	Activation of P2Y2 receptor; Inhibition of ectonucleotidases	Polyoxometalate $[Co_4(H_2O)_2(PW_9O_{34})_2]10-30$ (POM-5)	[151–154, 161]
Inhibition of Na^+ absorption; Increase in Cl^- secretion; Increase ASL height; Reduction of inflammation; Promotion of cell repair and migration	P2Y11 receptor; Fpr2 receptors; ATP	Activation	Lipoxin A4 (LXA4)	[53, 162–164]

signalization in ASL leads to the absence of purinergic-dependent-Cl^- secretion and the maintenance of Na^+ absorption by ENaC, causing mucus dehydration and decrease of ASL height. So, in the absence of functional CFTR, the purinergic system can be used to upregulate auxiliary Cl^- channels, as ANO1/TMEM16A, SLC26A9 or BK to

increase Cl^- secretion with the aim of restoring ASL homeostasis and mucus hydration by the stimuli of extracellular ATP. In this sense, the ANO1/TMEM16A upregulation could be an important CF therapeutic target, once ANO1/TMEM16A knockout mice showed a CF-like phenotype, which suggests the importance of this channel in Cl^- secretion [148].

Regarding the inflammation status in CF, the study by Anagnostopoulou et al. [145] compared the activity of SLC26A9 in wild type and SLC26A9-deficient mice by Th2-mediated inflammation by IL-13 treatment. The results suggest that in both groups the mucus overproduction has occurred; however, only in SLC26A9-deficient mice airway obstruction was observed, while in wild-type mice, Cl^- secretion was increased. However, Bertrand et al. [144] conclude that the activity of SLC26A9 as an anion channel in human bronchial epithelial (HBE) requires a functional CFTR. In addition, ASL homeostasis is regulated by a dominant extracellular purinergic signaling pathway that controls transepithelial ion flow [149, 150].

In this sense, the regulation of ASL dehydration occurs mainly by the action of two molecules, ATP and Ado (Fig. 1), that activate g-protein-coupled receptors, and regulate the conductance of ion channels in the apical cell membrane. The modulation of the signaling intensity basically occurs by water volume that regulates ATP and Ado concentrations. When ASL is dehydrated, the cilia–mucus interaction increases ATP and Ado concentrations, promoting the increase of ion conductance and consequently water volume. The increase in water volume, in its turn, reduces ATP and Ado concentrations and consequently, the signaling intensity [146].

Regarding the purinergic system, some specific receptors are attached to ASL volume regulation. One of these receptors is the P2Y2 receptor, which is stimulated by extracellular ATP. P2Y2 receptor stimulation results in the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2), via a phospholipase C-dependent mechanism, which causes the inhibition of Na^+ absorption by ENaC [151, 152]. On the other hand, PIP2 cleavage produces inositol 1,4,5-trifosfato (IP3), which causes an increase in intracellular Ca^{2+}, promoting Cl^- secretion by two channels, CaCC (ANO1/TMEM16A) and CFTR, besides generating K^+ efflux from BKs and basolateral membrane channel permeability (CaKCs) [153, 154]. However, the extracellular ATP is metabolized by ectonucleotidases located on the airway surface [155].

The ectonucleotidases can also be released in airway by epithelial or inflammatory cells via exosomes [156], or can be secreted by submucosal glands [157], and due to decrease of steady-state ATP levels, can contribute to ASL dehydration [120, 158, 159] by the absence of P2Y2 receptor stimuli by ATP, that could keep an alternative Cl^- secretion pathway.

In this sense, Sandefur et al. [24] developed a mathematical model of ASL homeostasis regulated by purinergic signaling, which is consistent with experimental observations from Tarran et al. [149], that have demonstrated that ATP and Ado are responsible for modulating the transport of ions Na^+ and Cl^- that regulate ASL height. This model showed that low concentrations of extracellular ATP and high concentrations of extracellular Ado promote decrease in Cl^- secretion and increase in Na^+ reabsorption, reducing ASL height, as observed in CF.

It is interesting to note that loss of lung functions in CF patients is also directly connected to exacerbations that can occur, for example, for viral infection, which impairs mucus transport [160]. The impairment of mucus transport, in this case, happens by virus-induced upregulation of ecto-ATPases that metabolize ATP, causing ASL depletion [149]. These data are presented by Sandefur et al. [24] to defend the importance of using the P2Y2 receptor/ATP signaling as a therapeutic target, once in their model, ASL height regulation in CF patients depends exclusively on this pathway.

Also aiming to identify ectonucleotidase inhibition as a therapeutic strategy to CF patients, Heusden et al. [161], studying human airway cells, have identified the polyoxometalate $[Co_4(H_2O)_2(PW_9O_{34})_2]^{10-}$ 30 (POM-5) as a potent and effective ectonucleotidases inhibitor, which increased steady-state levels of ATP and enhanced ASL volume.

In addition, another potential therapeutic strategy for CF is lipoxin A4 (LXA4), a molecule that inhibits Na^+ channels, restores [162] and/or increases ASL, stimulates calcium-activated chloride currents [163], in addition to playing an anti-inflammatory role [164, 165]. These effects are mediated by P2Y11 and formyl-peptide receptor 2 (FPR2) receptors. LXA4 stimulates the FPR2 channel, which leads to a release of ATP in apical cells, activating P2Y11 receptor. P2Y11 receptor stimulates Cl^- secretion and inhibits Na^+ absorption, thereby increasing ASL [53]. In addition, P2Y11 receptor activation also promotes cell repair and migration [164, 165]. However, there are still no clinical studies with LXA4 relating to CF, even though it is a very promising therapeutic target for the improvement of recurrent pulmonary aspects of this pathology, such as restoration of ionic transport in the epithelium bronchial, besides anti-inflammatory actions, tissue and functional repair of the epithelium and increased ASL height [53].

Conclusions

We showed for the first time that the reduction of Cl^- in CF secretion and the height of the ASL are regulated by ATP and Ado, through the P2Y2 and A2B receptors, playing an essential role in the generation of ionic fluxes. Thus, the purinergic system can promote the suprarregulation of

auxiliary Cl^- channels, increase the secretion of Cl^-, guaranteeing the homeostasis of the ASL, as well as the hydration of the mucus. Besides, Ado plays an important role in the anti-inflammatory response and also in the secretion of Cl^-, through the activation of A2A and A2B receptors. Then, the ADA blockade looks promising to increase Ado levels in CF.

The performance of the purinergic system in CF appears as an alternative to expensive CFTR modulating therapies, restricted to some patients with specific mutations, and ineffective for younger children, which excludes a significant portion of patients. To control the volume of ASL, high concentrations of ATP are required, and this can be achieved by modulating enzymes responsible for ATP hydrolysis, such as ENTPDases, ENPPs and NSAP. In addition, another possible therapeutic line would be the upregulation of P1 receptors and the blocking of P2 receptors. We postulate that the activation of A2A and A2B receptors and the inhibition of ectonucleotidases may eventually be used to contain the excessive inflammatory response of CF, as well as the use of antagonists acting at P2Y receptors (P2Y6, P2Y12 and P2Y14) and P2X7 receptor.

In conclusion, we emphasize the importance of basic research and clinical trials using the therapeutic potential of the purinergic system in CF. Bearing in mind that acute and chronic inflammation can be immune modulated by the purinergic system, it is expected that an association between the regulation of receptors and the functioning of the Cl^- channels will enable the development of treatments that can improve the quality of life of patients.

Author contributions TITdC had the idea for the article. TITdC, VEMS, FdA, JW, DTdReS, SFVdOM and MDB performed the literature search and data analysis, drafted and critically revised the work.

Funding No funding was received to assist with the preparation of this manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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