REVIEW

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NLRP3 inflammasome as a novel target for cystic fibrosis treatment

Merve Atalay, Başak Şen and Didem Dayangaç Erden[*]

Abstract

Background Inflammasomes are intracellular multiprotein complexes that sense danger signals from damaged cells and pathogens and assemble to mediate caspase-1 activation, which results in the proteolytic cleavage of pro-IL-1β and IL-18 into bioactive forms. The NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is a critical component of the innate immune system that mediates caspase-1 activation and secretion of the proinflammatory cytokines IL-1β/IL-18 in response to disturbances in cellular homeostasis caused by microbial infections and cellular damage.

Main body of abstract The NLRP3 inflammasome is associated with various inflammatory disorders, including Alzheimer's disease, diabetes, and atherosclerosis. In recent years, NLRP3 inflammasome has also been implicated in inflammation in cystic fibrosis. The differentiation of pro-IL-1β–IL-1β, an active cytokine, is mediated by neutrophil expression of the NLRP3 inflammasome. Furthermore, it maintains a cytokine storm in the lungs during the pathogenesis of CF.

Short conclusion This review highlights neutrophil metabolic reprogramming characterized by the Warburg effect, NLRP3-mediated inflammation in cystic fibrosis, and its inhibition as a potential therapeutic strategy.

Keywords Cystic fibrosis, NLRP3, Inflammation

Background

Cystic fibrosis is an autosomal recessive genetic disease caused by mutations in the Cystic Fibrosis Transmembrane Regulator (CFTR) gene. The CFTR gene has been mapped to 7q31.2 (Navarro 2016). Today, more than 2000 mutations have been identified in the CFTR gene which varies depending on ethnicity (The Clinical and Functional TRanslation of CFTR (CFTR2)). Mutations detected in Turkish population are genetically heterogeneous and different from European populations. In Europe, incidence rates ranging from 1:2800 to 1:10,000 have been reported, while incidence in Asia has greater variance between countries, ranging from 1:5800 in

Bahrain to 1:350,000 in Japan (Scotet et al. 2020). As a result of epidemiological studies conducted in central Turkey, the incidence of CF was estimated to be 2.9 per 10,000 live births (Hangul et al. 2019).

After CFTR protein is secreted from the endoplasmic reticulum, it is glycosylated in the Golgi body, reaches the apical membrane of epithelial cells, and acts as a mediator in the transport of chloride channels and bicarbonate ions (McClure et al. 2016). CFTR protein activated by cAMP and protein kinase A is responsible for the regulation of other chloride channels and epithelial sodium channels such as the epithelial sodium channel (ENaC) and Na^+/K^+ ATPase pumps. Along with fluid homeostasis on the epithelial surface, CFTR is also involved in the regulation of Na+ absorption and transport of small molecules such as glutathione (Tsui and Dorfman 2013). With the deterioration of ion balance in the airways of cystic fibrosis patients, an environment suitable for mucus accumulation and bacterial colonization is

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formed (Warrington et al. 2011). Failure of cilia to function properly and impaired mucociliary clearance cause inflammation and infections in the airway. The decrease in mucociliary clearance and the increase in IL-8 and IL-6 expressions indicate that one of the main pathways affected in epithelial cells is the inflammatory response pathway (Tsui and Dorfman 2013). The release of proinflammatory cytokines is thought to activate the NLRP3 inflammasome pathway in the cell by triggering the formation of necroptosome complexes responsible for activating the necroptosis pathway.

Pulmonary innate immunity

Innate immunity, one of the two immune system branches provides the first-line defense in host protection against infections. The oldest defense system in evolution, the innate immune system encompasses physical barriers along with cellular and humoral mediators (Warrington et al. 2011). Inflammation, the main mechanism of innate immunity, enables a rapid but non-specific response against pathogens. The defense cells involved in innate immunity (neutrophils, monocytes, macrophages, dendritic cells, and natural killer cells) can recognize pathogens and host cells through Pattern Recognition Receptors (PRRs) to trigger inflammation (Suresh and Mosser 2013).

Lung tissue is constantly exposed to pathogenic microorganisms in the environment, leading to lung infections or lung disease. The innate immune system, therefore, plays an important role as the first line of defense against pathogens in this vulnerable environment (Leiva-Juarez et al. 2018). However, some pathogens show partial resistance to innate immune mechanisms. The main pathogens of lung infection include *Streptococcus pneumonia, Staphylococcus aureus, Legionella pneumophila, Chlamydia pneumonia, Klebsiella pneumonia,* and *Pseudomonas aeruginosa* (Zhang et al. 2021). The airway epithelium is responsible for transporting air to and from the alveoli. It plays a crucial role in the lung's defense against infections and particles inhaled from the environment. In the pathogenesis of bacterial pneumonia, pulmonary resident innate immune cells [Airway Epithelial Cells (AECs), macrophages, and Dendritic Cells (DCs)] are critical (Kumar 2020).

Bronchial Epithelial Cells (BECs) and alveolar epithelial cells (AECs) serve as protective mechanical barriers against inhaled pathogens that cause pneumonia. AECs are divided into two types: type I AECs, which are primarily involved in facilitating gaseous exchange and may also recognize pathogens, and type II AECs, also known as type II pneumocytes and serve as innate immune cells (Eisele and Anderson 2011; Leiva-Juarez et al. 2018). Surfactant proteins which constitute the mucosal barrier are

also secreted on the apical surface of Type II pneumocytes. Type II AECs express basolaterally repair enzymes (fibrinogen or FBG), which respond swiftly to changes in osmotic pressure and recognize pore-forming poisons secreted by pathogenic bacteria (Guadiz et al. 1997).

Macrophages originate from the bone marrow and are formed by the differentiation of another immune system cell called monocytes. They settle in strategic spots where microorganisms can enter the body, quickly recognize strangers, and respond with a lethal response. These cells, which are like neutrophils, stay longer at the site of inflammation and have a longer lifespan than neutrophils (Byrne et al. 2015). Another feature that distinguishes them from neutrophils is that they play a regulating role in inflammation with the chemical stimuli they secrete, called cytokines, and provide the initiation of specific/acquired immunity. Important cytokines secreted from macrophages include IL-1 (Interleukin 1), IL-6, complement proteins, hydrolytic enzymes, Interferon-alpha (IFN-α), and Tumor Necrosis Factor-alpha (TNF-α) (Kumar 2020). Dendritic cells are responsible for initiating the immune response by presenting pathogens, to other immune system cells. It can be regarded as the first step in the initiation of the specific pulmonary immune response (Peters et al. 2019).

The defense cells mentioned above use their surface receptors to distinguish between friend and foe. Pathogens, such as bacteria, form their species-specific molecular structures on their surfaces. These molecules are called Pathogen-Associated Molecular Patterns (PAMPs). The structural feature of these molecular motifs has been conserved throughout evolution and can be detected by PRRs. PRRs also recognize molecular markers of host tissue damage named Damage-Associated Molecular Patterns (DAMP). NLRP3 which recognizes PAMPs and DAMPs activates caspase-1 in the inflammasome, resulting in the release of IL-1β and IL-18 cytokines (Kim et al. 2016).

Concept and structure of NLRP3 inflammasome

Inflammasomes are multimeric protein complexes that act as platforms for caspase activation and the eventual release of pro-inflammatory cytokines. Activated inflammasomes lead to pro-inflammatory cytokine release and Gasdermin D-dependent pyroptotic cell death, enabling the activation of important defense mechanisms such as cell migration (Akbaba et al. 2022) and NET formation (de Torre-Minguela et al. 2017; Sollberger et al. 2018; Pandey et al. 2021). NLRP3 is one of the inflammasomes expressed in dendritic cells, monocytes, macrophages, neutrophils, and epithelial cells (Kummer et al. 2007; Guarda et al. 2011).

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NLRP3 inflammasome activation requires the integration of two signals, referred to as Signal 1 and Signal 2. Signal 1 is triggered by PRRs such as Toll-like receptor 4 (TLR4) and results in the priming and licensing of NLRP3. Priming refers to the NF-κB-mediated transcription of NLRP3, pro-IL-1β, and pro-IL-18. Licensing involves the regulation of post-translational modifications, protein–protein interactions and cellular localization of NLRP3 that can be used to modulate its activity. Signal 2 can be triggered by many events that lead to disruption of cellular homeostasis and result in the assembly of the inflammasome complex. Various PAMPs and DAMPs have been shown to activate the inflammasome, such as extracellular ATP, pore-forming toxins and viral RNA (Paik et al. 2021). These stimuli activate NLRP3 indirectly by causing perturbations in cellular homeostasis. Events such as K^+ and Cl^- efflux, Ca^{++} influx, excessive mitochondrial ROS production, lysosomal damage (Paik et al. 2021), and ER stress (Menu et al. 2012) are among these perturbations.

Upon activation, NLRP3 oligomerizes, which allows for interaction with the ASC adapter protein and recruitment of pro-caspase-1. This recruitment allows for the proximity-induced self-cleavage and activation of caspase-1 (Wang and Hauenstein 2020), which itself cleaves the cytokine precursors pro-IL-1β and pro-IL-18. Ultimately, the activation of the NLRP3 inflammasome leads to the release of these pro-inflammatory cytokines (Fig. 1).

IL-1 is a major player in the initiation of acute inflammation. Locally, IL-1 signaling controls the recruitment and stimulation of neutrophils and macrophages, the release of more inflammatory mediators (e.g., prostaglandins, nitric oxide, metalloproteinases), and the

Fig. 1 A model of NLRP3 inflammasome activation. *DAMPs* Damage-associated molecular patterns *PAMPs* Pathogen-associated molecular patterns

differentiation of lymphoid cells. Systemically, IL-1 acts to induce fever, hypotension, neutrophilia and activate the acute phase reaction (Sollberger et al. 2018; Chan and Schroder 2020). IL-1 can also induce its production in an autocrine/paracrine manner, playing a role in the maintenance of inflammation (Dinarello et al. 1987). This inflammatory response is integral in the defense against many pathogens; however, sustained and excessive inflammation can be detrimental to the host. In the lungs of patients with CF, chronic inflammation characterized by neutrophilia can be observed. Accordingly, concentrations of IL-1β in bronchoalveolar lavage (BAL) fluid are increased, especially in the context of infection (Bonfield et al. 1995; Montgomery et al. 2018). This persistent inflammatory state that is ineffective at controlling infections eventually results in airway damage and remodeling of the lung tissue. The resulting bronchiectasis leads to pulmonary function decline, which is a major cause of morbidity and mortality in patients with CF (Balazs and Mall 2019).

The NLRP3 inflammasome promotes inflammation not only in the context of infection but also in many conditions that lead to disruptions in homeostasis. NLRP3-mediated inflammation has been linked to the pathogenesis of many diseases including autoimmune conditions such as rheumatoid arthritis, systemic lupus erythematosus (Li et al. 2020); neurodegenerative diseases such as Parkinson's and Alzheimer's, Huntington's disease (Holbrook et al. 2021); and cardiovascular diseases such as atherosclerosis and hypertension (Toldo et al. 2021).

Main text
NLRP3-mediated inflammation in cystic fibrosis
In cystic fibrosis, excessive activation of neutrophil-mediated innate immunity is a major driver of lung disease progression (Tucker et al. 2021). Recent evidence suggests that in these patients, an intrinsic tendency for hyper-inflammation may be linked to mutant CFTR function independent of the presence of infection (Cantin 2019). The NLRP3 inflammasome emerges as the potential primary driver of this tendency since it can be directly activated by disruptions of homeostasis not necessarily caused by pathogens (Bai et al. 2020). NLRP3-mediated inflammation has been proposed to be induced by many mechanisms, including changes in immunometabolism such as the Warburg effect, disturbances of intracellular ionic concentrations, mitochondrial dysfunction and ER stress (Cantin 2019). The interplay between CF inflammation and NLRP3 activation caused by these mechanisms is reviewed below.

The Warburg effect is characterized by increased glucose uptake, increased aerobic glycolysis, increased
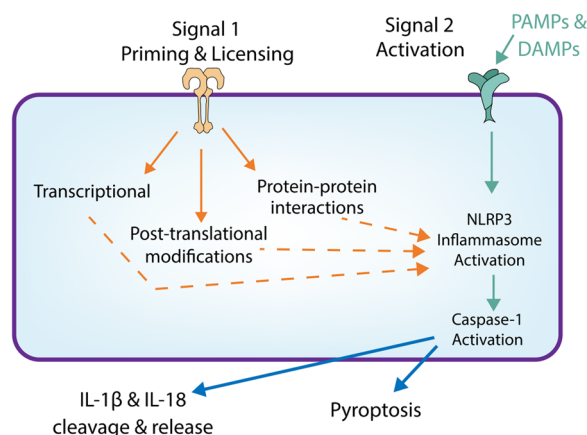
lactic acid production, the resistance of glucose entering the Krebs (tricarboxylic acid) cycle and ATP accumulation (Mahla et al. 2021). Improved aerobic glycolysis in cells is mainly due to the increased expression of key enzymes in glycolysis, most of which are regulated by hypoxia-inducible factor-1α (HIF-1α) (Prakasam et al. 2018). The lactic acid that is the product of the Warburg effect, can harm the body. Studies have shown that lactic acid is transported into the extracellular environment by monocarboxylic acid transporter protein, which causes acidification of the microenvironment and inflammatory reactions (Marchiq and Pouyssegur 2016). The disruption of mitochondrial metabolism and increased aerobic glycolysis is evidence of cell tumorigenesis (Cassim et al. 2020). Therefore, the inflammatory response might be related to the increased Warburg effect which is observed in malignancy under normal conditions, and which can also be triggered by CFTR defect in neutrophils.

The CF airway environmental signals known as DAMPs and PAMPs such as bacterial LPS, affect neutrophil immunometabolism. In neutrophils, the transcription of PKM2 (pyruvate kinase M2 isoform), a critical metabolic protein, is increased after being activated by LPS. PKM2 undergoes post-translational modification and forms a transcriptional complex with PHD3, P300, and HIF1 (hypoxia-inducible factor-1) to trigger many glycolytic proteins (Prakasam et al. 2018). High PKM2, lactate, succinate levels, and low pH provide additional evidence for the presence of the Warburg effect in CF neutrophils (Fig. 2). The Warburg effect is a reprogramming of neutrophil glucose metabolism toward glycolysis rather than oxidative phosphorylation via the Krebs cycle. The HIF1 complex binds to the HRE (HIF1-responsive element) in neutrophils, causing the increased production of PKM2, HIF1, and importantly pro-IL-1β. This increased pro-IL-1β was shown to be predominantly processed by the NLRP3 inflammasome before being secreted (Liberti and
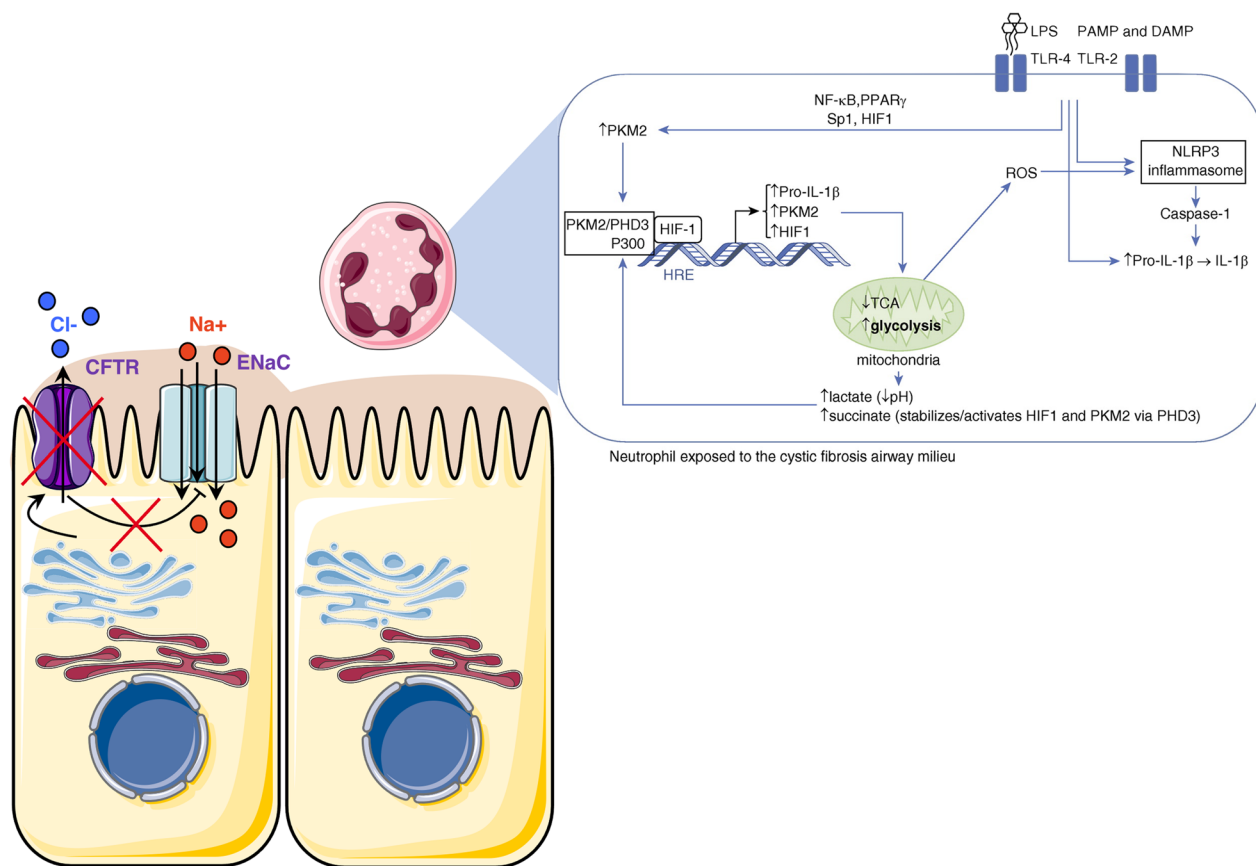
Fig. 2 Immunometabolism of neutrophils in cystic fibrosis. Irregularity in the transport of Na$^+$ and Cl$^-$ in the lung epithelial cells of patients with CF is shown in the figure. In addition, an inflammation originating from PAMPs and DAMPs begins in neutrophils in lung cells, where the CFTR protein cannot show proper activity. This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. Figure inset from (Cantin 2019) licensed under Creative Commons Attribution, Non-Commercial, No Derivatives License 4.0. *ENaC* epithelial sodium channel, *DAMPs* Damage-associated molecular patterns, *NF-κB* Nuclear factor-κB, *PAMPs* Pathogen-associated molecular patterns, *PPARγ* Peroxisome proliferator-activated receptor-γ, *TLR* Toll-like receptors

Locasale 2016). During its encounter with the CF lung environment, neutrophils undergo significant alterations in immunometabolism which include the Warburg effect and these changes have an impact on the cell's ability to promote NLRP3-mediated IL-1β-dependent inflammation, a cytokine that is closely linked to poor patient outcomes (McElvaney et al. 2019).

In CF, mutant CFTR leads to the upregulated expression of the epithelial sodium channel ENaC (Bangel et al. 2008), leading to the disruption of Na$^+$ and K$^+$ homeostasis in addition to Cl$^-$. Since K$^+$ efflux is a well-known trigger for NLRP3 activation, Scambler et al. investigated a possible connection between inflammasome activation and ionic disturbances caused by mutant CFTR (Scambler et al. 2019). They found that both bronchial epithelial cells and monocytes from CF patients showed increased NLRP3 activation resulting in increased secretion of IL-1β and IL-18. The activation of NLRP3 was dependent on the increased activity of ENaC and could be reversed by its inhibition. They, therefore, proposed a mechanism in which increased Na$^+$ influx and the resulting K$^+$ efflux in CF cells trigger the activation of the NLRP3 inflammasome. This study, therefore, provides evidence linking the primary pathophysiology in CF to a pro-inflammatory tendency.

Ianitti et al. used both mice and human CF models to investigate the contribution of different inflammasomes in the context of infection (Iannitti et al. 2016). They found that NLRP3 expression and IL-1β expression were higher and more persistent in CF models compared to control, and this increased NLRP3 activity was associated with increased susceptibility and higher mortality with *Pseudomonas aeruginosa* and *Aspergillus* infection. Interestingly, NLRP3 knockout mice were shown to be more resistant to either infection, despite the presumed protective activity of innate immunity. These results suggest that inflammation in CF patients may be detrimental to the control of infections as opposed to healthy people, where inflammation is an important first-line defense.

Another study conducted by Rimessi et al. demonstrated a mitochondria-mediated Ca^{++} signal in *P. aeruginosa* infected cells that could not be corrected by mutant CFTR (Rimessi et al. 2015). This intracellular Ca^{++} resulted in the activation of NLRP3. Although it has not been demonstrated directly, another possible mechanism of NLRP3 activation in CF is through ER stress. The accumulation of misfolded CFTR protein was shown to result in an inflammatory response in CF macrophages (Lara-Reyna et al. 2019) and this pathway was linked to the NLRP3 inflammasome in an ER stress model (Bronner et al. 2015).

Taken together, numerous studies suggest that in CF, NLRP3-mediated inflammation is triggered by multiple mechanisms dependent on the clinical context. As this inflammation may not be necessary to control infection and in fact, may even be detrimental to patients; NLRP3 emerges as a novel target in cystic fibrosis. Different approaches for NLRP3 inhibition and their applications in CF are summarized below.

NLRP3 inhibitors as new therapeutic strategy in cystic fibrosis

MCC950

MCC950, first identified as a specific inhibitor of NLRP3 in 2015 (Coll et al. 2015), blocks canonical, non-canonical, and alternative activation of the NLRP3 inflammasome. By inhibiting the ability of NLRP3 to hydrolyze ATP, it blocks NLRP3 from assuming its open conformation (Tapia-Abellan et al. 2019) and the ATP-dependent oligomerization of the inflammasome. (Coll et al. 2015).

McElvaney et al. showed that treatment of CF mice infected with *P. aeruginosa* with intraperitoneal MCC950, resulted in reduced IL-1β concentrations in the lung and improved bacterial clearance. (McElvaney et al. 2019) As excessive inflammation can result in ineffective infection control in CF patients, this study hints at the potential of targeting NLRP3 as a therapeutic strategy. In the same study, wild-type mice exposed to LPS were also treated with MCC950. Interestingly, administration of MCC950 before LPS exposure resulted in a more substantial reduction of IL-1β levels than post-exposure administration. This represents that MCC950 can have a prophylactic as well as therapeutic effect, possibly by preconditioning neutrophils in systemic circulation before they are primed by LPS. Recently, Clauzure et al. used a CF bronchial cell-based model to examine the connection between CFTR dysfunction and NLRP3 activation (Clauzure et al. 2021). They were able to demonstrate that increased intracellular Cl$^-$ levels led to the increased expression and release of IL-1β and these effects were diminished with MCC950. As well as providing a potential mechanistic explanation for NLRP3 activation in CF, this study also serves as another demonstration of MCC950 as a potential therapeutic agent in this disease. Overall, both studies support the possible use of specific inhibition of the NLRP3 inflammasome as an anti-inflammatory strategy in CF.

Anakinra

Anakinra, a recombinant form of the endogenously produced IL-1 receptor antagonist (IL-1Ra), is an anti-inflammatory drug used to inhibit IL-1 signaling in a wide variety of inflammatory conditions such as rheumatoid arthritis, recurrent pericarditis, and autoinflammatory syndromes (Dinarello et al. 2012).

Ianitti et al. showed that treatment of *P. aeruginosa* infected CF mice with anakinra resulted in reduced bacterial burden, neutrophil recruitment and histological lung damage (Iannitti et al. 2016). Anakinra reduced the half-life of NLRP3 via the ubiquitin–proteasome system. In in vitro experiments, Ianitti et al. showed that after treatment with anakinra, IL-1β expression and NLRP3 staining were decreased in human CF bronchial epithelial cells, in response to infection with *A. fumigatus* and *P. aeruginosa*, compared to healthy controls. In addition, anakinra treatment of CFTR knockout mice led to increased survival and reduced lung damage in the context of *Aspergillus* and *Pseudomonas* infections. Interestingly, endogenous production of IL-1Ra was impaired in CF bronchial epithelial cells and the sputum of CF patients, suggesting a defect in the counter-regulation of NLRP3 activity. Altogether, these findings imply that IL-1 signaling is dysregulated in CF and therefore can be targeted as an additional anti-inflammatory strategy.

Another study, performed by Pariano et al. showed that Superoxide Dismutase 2 (SOD2) expression, which was reduced in CF mice compared to healthy controls, was increased with anakinra treatment (Pariano et al. 2021). SOD2 is an antioxidant enzyme that acts as a scavenger of mtROS, an important activator of NLRP3. siRNA knockdown of SOD2 in non-CF mice infected with *A. fumigatus* led to increased expression of IL-1α and IL-1β while abrogating the therapeutic effects of anakinra in infection. Taken together, these results suggest that anakinra can also indirectly inhibit NLRP3 activity through its effects on ROS metabolism. Although the mechanism of action of anakinra most likely expands beyond its direct and indirect effects on the NLRP3 inflammasome, it nevertheless emerges as a potential anti-inflammatory treatment in CF.

IL-37

IL-37 is a cytokine of the IL-1 family with anti-inflammatory effects (Dinarello 2018). In a mouse aspergillosis model, Moretti et al. showed that IL-37 reversed the increased expression of NLRP3 in response to infection. This effect was mediated by the IL-1 family decoy receptor Single Ig And TIR Domain Containing (TIR-8/SIGIRR) (Moretti et al. 2014). IL-1β production was also decreased as a result. IL-37 treatment of CFTR-deficient mice with aspergillosis led to decreased recruitment of neutrophils to the lungs. Similar effects were detected in *Aspergillus-infected* NLRP3-deficient mice which weren't modified by IL-37 treatment. In another non-CF model, the decrease in NLRP3 expression was not observed in IL-37 expressing mice (Rudloff et al. 2020). However, IL-37 ultimately resulted in decreased ASC speck formation and caspase-1 formation. Although both studies

have demonstrated inhibition of NLRP3-mediated IL-1 production by IL-37, they propose different mechanisms of action. This discrepancy points to the need for more research on the anti-inflammatory actions of IL-37 as well as other endogenous anti-inflammatory molecules. Further studies may pave the way for potential recombinant agents to be used in CF therapy.

Conclusions

The NLRP3 inflammasome has been the subject of most research during the last decade. However, the field has yet to find out a consistent mechanism for NLRP3 inflammasome activation. Multiple signaling and biological events are induced by NLRP3 stimuli, which are demonstrated to activate the NLRP3 inflammasome. The expression of NLRP3 inflammasome in neutrophils affects cytokine storm and differentiation of pro-IL1β–IL1β which is an active cytokine. In addition, it maintains the cytokine storm in the lungs in CF pathogenesis. As a result, by evaluating inflammation activated in respiratory diseases such as cystic fibrosis in terms of NLRP3 inflammasome structure and NLRP3 inhibitor molecules, it may be possible to elucidate the molecular basis of the diseases and determine new treatment strategies.

Abbreviations

AEC	Airway epithelial cells
ASC	Adaptor molecule apoptosis-associated speck-like protein containing A CARD
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
DAMP	Danger-associated molecular patterns
DC	Dendritic cell
ENaC	Epithelial sodium channels
ER	Endoplasmic reticulum
FBG	Fibrinogen
HIF-1	Hypoxia-inducible factor-1
HRE	HIF1-responsive element
IFN-α	Interferon alpha
IL-1	Interleukine 1
IL-1β	Interleukine 1 beta
IL-18	Interleukine 18
IL-1Ra	IL-1 receptor antagonist
IL-37	Interleukine 37
LPS	Lipopolysaccharide
NF-κB	Nuclear factor kappa B
NLRP3	NLR family pyrin domain containing 3
PAMPs	Pathogen-associated molecular patterns
PHD3	Prolyl hydroxylase 3
PKM2	Pyruvate kinase M2
pro-IL-1β	Pro-inflammatory cytokine IL-1β
PRR	Pattern recognition receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen Species
siRNA	Small interfering RNA
SOD2	Superoxide dismutase 2
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha

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