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Mapping Targetable Inflammation and Outcomes with Cystic Fibrosis Biomarkers

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Summary

Cystic fibrosis is characterized by an overly exuberant neutrophilic inflammatory response to pathogens and other stimuli that starts very early in disease. The overwhelming nature of this response is a primary cause of remodeling and destruction of the airways, suggesting that anti-inflammatory therapies could be beneficial in CF. However, finding therapies that can effectively reduce the inflammatory response without compromising host defenses remains elusive. New approaches towards mapping inflammatory targets promise to aid in developing novel therapeutic strategies and improve outcomes in individuals with CF.

Keywords

Sputum; bronchoalveolar lavage; exhaled breath condensate; metabolomics

Introduction

Inflammation in cystic fibrosis (CF) is characterized by a marked and persistent influx of neutrophils into the airways. Despite the overwhelming nature of this inflammatory response, it remains insufficient to eradicate infection, resulting in a vicious cycle of infection, inflammation, and mucus hypersecretion/dehydration that causes progressive remodeling and destruction of the airways. This high degree of airway inflammation is responsible for much of the lung disease in CF, with concentrations of inflammatory biomarkers (particularly neutrophil elastase) the most predictive of disease progression[1,2]. Nevertheless, there are relatively few therapies developed to directly address airway inflammation in CF. This lack of treatment options reflects several challenges in developing effective anti-inflammatory therapies, including difficulties in measuring airway inflammation. This review will summarize the origins of airway inflammation in CF, current options for treatment, and how developments in measuring biomarkers of airway inflammation may lead to a new generation of anti-inflammatory treatments for CF.

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Origins of CF Inflammation

A key finding of studies of early CF lung disease is that airway inflammation begins at or very soon after birth. Neutrophils and neutrophil elastase can be detected in bronchoalveolar lavage (BAL) in patients diagnosed with CF by newborn screen as early as 3 months of age, and these inflammatory markers correlate with future development of bronchiectasis and gas trapping on CT scan[1]. This increase in inflammation does not appear to be solely a response to infection, since less than half of infants with neutrophil elastase detected in BAL fluid had an active pulmonary infection or history suggesting infection. These observations suggest that inflammation in CF airways is multifactorial (Figure 1) and can occur even in the absence of an infectious stimulus.

Localized hypoxia in the CF lung could explain the early inflammation seen in the absence of obvious infection[3]. The gene mutated in CF, the cystic fibrosis transmembrane conductance regulator (*CFTR)*, encodes a cAMP dependent anion channel that conducts chloride and bicarbonate and regulates the balance of chloride secretion and sodium absorption in the airway.[4] Loss of CFTR channel activity produces a dehydrated airway surface environment where the total mass of salt and volume of water are inadequate to maintain mucus hydration, leading to defects in mucociliary clearance. The resulting thickened mucus and mucus plugging in the small airway create localized areas of hypoxia, which can trigger inflammatory responses[5] including release of cytokines such as IL-1 and activation of the inflammatory cascade via binding to the IL-1 receptor[6]. The resultant increase in inflammation may then worsen hypoxia and contribute to a niche for anaerobic bacteria, thus further propagating the inflammatory cycle[3].

While infection may not be required to initiate inflammation in CF, defects in immune responses to pathogens likely contribute to the excessive inflammatory environment. A number of mechanisms of immune dysregulation have been described in CF, including aberrant responses in inflammatory cells such as neutrophils and macrophages as well as altered signaling pathways in airway epithelia. These aspects of immune dysregulation were reviewed in detail in 2015 by Nichols and Chmiel in a previous volume of “Barriers to Normalcy”[7] and will be only briefly summarized here. The mucus dehydration and impaired mucociliary clearance contribute to enhanced inflammatory responses, with failure to clear pathogens out of the airway leading to prolonged stimulation of inflammatory pathways[8,9]. Furthermore, there is evidence that CFTR may play a more direct role in regulation of inflammatory responses. For example, neutrophils isolated from patients with CF tend to undergo necrotic rather than apoptotic responses, releasing additional pro-inflammatory molecules such as High Mobility Group Box 1 (*HMGB1*) protein and metalloproteases[10,11]. There is also evidence that CFTR is involved in the acidification of phagosomes and bacterial killing in both neutrophils and macrophages[12,13]. Similarly, CF macrophages and monocytes also demonstrate defective immune response[14].

Studies of animal models suggest that defects in innate immunity contribute to the excessive inflammatory responses in CF. CF pigs have decreased bacterial clearance and increased inflammation relative to unaffected litter mates after exposure to bacterial pathogens[15]. CFTR knockout ferrets[16] also show abnormal bacterial clearance and enhanced

inflammatory responses[17]. The mechanisms that underlie these defects are not fully defined, though there is evidence that defective bacterial clearance in the CF pig reflects altered airway acidification, likely related to loss of CFTR mediated bicarbonate secretion that alters the efficacy of antimicrobial peptides[18].

Despite this evidence, the role of altered inflammatory responses directly related to CFTR deficiency (as opposed to secondary effects from defective mucociliary clearance) remains controversial. Systemic infection remains uncommon in CF despite high airway bacterial loads[19], raising some questions about the clinical relevance of abnormalities observed in isolated CF inflammatory cells. Studies in animal models must also be interpreted with caution, since no animal model faithfully recapitulates all aspects of human disease. For example, the altered airway pH observed in pigs may not be present in human CF[20], and airway acidification similar in magnitude to that of the CF pigs has been observed in asthma[21,22], a disease that is not commonly associated with airway infection[21,22].

Anti-inflammatory therapies in CF

Although the factors that contribute to inflammation in CF are not fully defined, the relevance of inflammation as a therapeutic target is unquestioned[23]. Nevertheless, despite intensive effort, limited therapies are available. Prednisone is perhaps the most canonical anti-inflammatory, and alternate day therapy with prednisone has been shown to increase forced vital capacity (FVC) in treated CF patients compared to placebo[24]. However, chronic use of systemic steroids is contraindicated due to their adverse effects including growth retardation, osteoporosis, cataracts, hyperglycemia and risk of opportunistic infection. High dose ibuprofen is a more targeted anti-inflammatory that has been shown to slow the rate of decline of FEV_1 in two separate double blind, placebo controlled studies[25,26], and this clinical benefit has been associated with a decrease in neutrophil migration to the lung[27]. Although trials with ibuprofen did not show a significant increase in adverse events between treatment and placebo groups, the perceived risk of gastrointestinal bleeding and renal toxicity coupled with the need to obtain serum levels to minimize these risks has inhibited widespread use of this drug.

The most widely used therapy in CF with anti-inflammatory properties is azithromycin. Interest in azithromycin as a CF therapeutic stemmed from its benefit in diffuse panbronchiolitis[28], a disease with many similarities to CF, and was thought to possibly relate to its antimicrobial activity against *Pseudomonas aeruginosa* growing in biofilms[29]. Indeed, the initial large study of chronic, low dose azithromycin in CF was targeted towards patients with persistent *Pseudomonas* infection. This study demonstrated that chronic azithromycin treatment led to improvement in FEV_1, a decrease in exacerbations requiring antibiotic therapy, as well as improved quality of life (QOL) scores[30,31]. However, the clinical benefits occurred despite minimal impact on *Pseudomonas* bacterial density, suggesting that a different mechanism of action was responsible. Azithromycin has a number of anti-inflammatory effects, including reduction in neutrophil oxidative burst and increases neutrophil apoptosis[32,33]. In lung macrophages azithromycin also appears to inhibit apoptosis, stimulate phagocytosis of bacteria and cellular debris, as well as skew macrophage cytokine expression toward an anti-inflammatory phenotype[34]. Other anti-

inflammatory effects of azithromycin include decreased mucin production with a resultant decrease in mucus viscosity, maintenance of tight junctions between epithelial cells and improvement of the integrity of the epithelial cell layer under inflammatory conditions[33]. These immunomodulatory effects may underlie the benefits of azithromycin more than its anti-Pseudomonal activity, and a large multi-center study demonstrated clinical benefit of chronic azithromycin in patients who did not have *Pseudomonas* infection[35].

Given the extensive number of pathways identified as playing roles in CF airway inflammation, it may seem surprising that other anti-inflammatory therapies have not yet been developed. However, an effective anti-inflammatory for CF must manage a careful balancing act, providing sufficient potency to reduce inflammation induced lung damage without interfering with the ability to resolve infection. This balance can be difficult to achieve, as revealed by the Phase II clinical trial of BIIL 284 BS, a promising antagonist of the leukotriene B4 receptor known to play a significant role in CF airway inflammation. This trial was stopped early due to an increase in pulmonary adverse events in those receiving the active drug compared to placebo[36]. Further studies showed that treatment of CF mice with BIIL 284 BS interfered with their ability to resolve *Pseudomonas aeruginosa* respiratory infection[37]. These results showcase the difficulty of balancing a reduction in inflammation while not significantly increasing the bacterial burden with the use of anti-inflammatory therapies and emphasize the need for pre-clinical testing of novel therapeutics[23].

Barriers to anti-inflammatory development: measuring airway inflammation

The dearth of effective anti-inflammatory therapies represents an ongoing barrier to normalcy in CF and suggests a need to identify new therapeutic targets. Testing of anti-inflammatory therapies and other treatments in CF has become increasingly challenging, as overall improvements in lung function and health make observing changes in traditional endpoints such as lung function or pulmonary exacerbations more difficult to assess without large and expensive trials[38]. However, accurately measuring airway inflammation directly as a marker of therapeutic activity can be difficult. Indeed, most of the major trials of anti-inflammatory therapies described above[24–26,36] did not include an airway inflammation biomarker, with the exception of the azithromycin trial that demonstrated statistically significant though modest changes in neutrophil elastase[30]. Although treatment related reductions in airway inflammation biomarkers were often shown in smaller studies[27,39], better biomarkers of airway inflammation are clearly needed to identify potential therapeutic targets and serve as surrogate markers of efficacy for clinical trials. This need is particularly great in young children to try and limit inflammation before the onset of lung damage. The challenges in developing better biomarkers reflect limitations in the primary methods to obtain airway samples: sputum collection, bronchoalveolar lavage, and assessments of exhaled breath (Figure 2, Table 2).

Sputum

Historically, assessments of airway inflammation in CF (and other diseases) have been primarily based on analysis of biomarkers in sputum, in part reflecting the long experience and existence of standardized protocols for this airway sample. Given the intense airway

inflammation that characterizes CF, it is no surprise that a multitude of inflammatory biomarkers are elevated in CF sputum, as summarized in several excellent reviews[2,40]. Among these biomarkers, sputum neutrophil elastase has emerged as one of the most predictive, with concentrations of sputum NE most highly correlated with lung function decline in large studies[2,41,42].

However, the utility of sputum is limited somewhat by the need for specialized procedures to process samples that typically must be performed immediately after collection[43]. Spontaneously expectorated sputum likely arises from more affected regions of the lung, and concentrations of inflammatory markers can be influenced by regional variability in lung disease[39] Furthermore, in general only older patients with more advanced disease can regularly expectorate sputum spontaneously. While sputum induction using hypertonic saline can be utilized to obtain samples from patients who do not spontaneously expectorate, many younger children have difficulty expectorating sputum even after induction[44,45]. Thus, sputum has a limited role in assessing—and by extension treating—airway inflammation in the youngest children.

Bronchoalveolar lavage fluid

For patients who cannot expectorate sputum, flexible bronchoscopy with BAL is considered the gold standard for airway biomarker assessment[40]. As with sputum, numerous inflammatory biomarkers are elevated in BAL fluid in children with CF including neutrophil counts, neutrophil elastase, pro-inflammatory cytokines such as interleukin-8, and others[46–51]. Several of these inflammatory biomarkers correlate with other aspects of disease severity including infection[52], radiologic findings[51,53,54], and infant lung function testing[46,49]. Like sputum, neutrophil elastase represents one of the most informative markers in BAL fluid, with elevated concentrations in infancy predictive of future bronchiectasis [1,55–57].

Use of BAL fluid as a source of airway inflammation biomarkers is constrained by several limitations, including the time, expertise, and expense needed for the procedure[58]. Furthermore, bronchoscopy requires sedation, which carries both short term risks and increasing concerns about long term adverse outcomes [59]. Due to these limitations, BAL has seen a limited role in clinical trials, though longer term observational studies that include BAL biomarkers such as AREST CF have provided significant insights into early disease[51,60,61].

Exhaled breath

Many of the limitations of sputum and BAL can be overcome through use of exhaled breath, which contains both volatile and non-volatile compounds that could serve as inflammatory biomarkers. Exhaled biomarkers are often collected as exhaled breath condensate (EBC), and since collection only requires the subject to exhale through a chilled tube, EBC can be obtained simply and non-invasively even in young children[62,63]. Indeed, a number of airway inflammatory biomarkers that are informative in sputum or BAL fluid are also elevated in EBC from subjects with CF, including inflammatory cytokines[63–66], 8-isoprostane[64,67], nitrates[64,68], leukotrienes[69], and purines[49,70], with measures of EBC leukotrienes and

purines shown to track changes related to CF exacerbations[69,70]. EBC pH has also been shown to be decreased in subjects with CF and change with treatment of an exacerbation[71–73]. With specialized methods, EBC can even be collected from the youngest children during infant pulmonary function testing (iPFTs)[74–76].

The ease of EBC collection is belied by difficulty in analysis, with EBC being described as "easy on patients" but "hard on scientists"[77]. Airway secretions in EBC arise from microaerosols generated during respiration, which represent a very low and highly variable fraction of the fluid volume of the condensate and may under-sample obstructed airways[78,79]. Therefore, extremely sensitive methods are typically needed to assess the low concentrations of most traditional biomarkers found in EBC, which ideally should also include a means to control for variable dilution[78,80]. Our own approach has been to utilize mass spectrometry to measure relevant biomarkers as well as urea as a dilution marker[81–83], though there are other valid methods[30,32]. Failure to adequately address these challenges impacts the reproducibility and validity of EBC biomarkers and may limit their utility as effective measures of airway inflammation[84].

Some of the limitations of EBC can be addressed by a focus on volatile biomarkers, which are not dependent on microaerosol generation for incorporation in exhaled breath. Several studies had shown that volatile organic carbon (VOC) profiles are altered in individuals with CF and could serve as inflammatory biomarkers[85,86]. One of the potentially exciting application of VOC profiling in CF is the development of electronic "nose" systems that could provide information on airway inflammation at the point of care[87,88]. However, current methods require sophisticated mathematical modeling to identify complex patterns in the detected VOCs, and the reproducibility of these signatures and their relationships to specific aspects of airway inflammation have not been established.

Non airway samples

The high levels of inflammation in the airways of individuals with CF translate into increases in systemic inflammatory biomarkers that could be assessed in serum or plasma, which are relatively easily obtained and analyze. Indeed, a large number of blood inflammatory markers are elevated relative in CF, including C-reactive protein[89–92], immunoglobulin G[90,93,94], cytokines[91], tumor necrosis factor[95], and transforming growth factor β[96], many of which are altered with pulmonary exacerbation[91,92,96,97]. However, the potential contribution of non-pulmonary inflammation reduces the specificity of these biomarker for lung disease and limits applicability. There have also been small trials investigating the use of biomarkers in both saliva and urine as a surrogate for lung inflammation[98,99].

Imaging

A number of small studies have been done using fluorodeoxyglucose (FDG) PET to quantify lung inflammation and follow response to treatment of CF exacerbations. FDG is concentrated in activated neutrophils which are recruited to sites of inflammation. The degree of inflammation can be estimated by the degree of FDG emission. A study following the kinetics of FDG movement to the lung showed that increased influx into the lung

correlated with a more rapid decline in FEV1 over time[100]. Other studies have used FDG PET monitor changes in inflammation during antibiotic treatment of CF exacerbation. Patients underwent FDG PET on days 1 and 14 of treatment and degree of inflammation was determined using standard uptake values (SUV). This group found that over the course of 14 days of IV therapy the max SUV decreased[101]. Regular use of FDG PET CT is limited by radiation exposure, however, as low dose CT protocols improve this may become a useful technique to follow lung inflammation.

Novel strategies

Despite all we have learned about the inflammatory pathways involved in CF lung disease, numerous challenges remain in translating these findings into effective anti-inflammatories. Many signaling pathways are not easily amenable to pharmacological inhibition, often requiring biologic antibody based treatments that while effective can be expensive and difficult to administer[102]. Other pathways may be too vital to host defenses to serve as a viable therapeutic targets, as suggested by the outcomes of the BIIL 284 BS study[36]. Therefore, there remains an urgent need to identify new pathways that could serve as viable targets for anti-inflammatory development. The ideal pathway would have defined characteristics, including an involvement in early disease, a readily measurable biomarker of activity, and availability of a relatively simple pharmaceutical treatment. Perhaps most importantly, blockade of this pathway should reduce inflammation without interfering with the ability to resolve infection (Table 1).

Use of ‘omics strategies, particularly metabolomics, is well suited towards identifying pathways that meet these criteria. The changes in metabolite patterns associated with disease reflect cellular enzymatic activities, which are attractive as therapeutic targets since they can often be inhibited by small molecule therapeutics[103]. Furthermore, the identified metabolites can serve as biomarkers of pathway activity and drug effects, many of which can be readily measured using standard methods even in non-invasive sample such as EBC[81,82,104]. The potential metabolomics has been demonstrated in several studies that find CF specific metabolite patterns in sputum[105], BALF[106–108], blood[109,110], and even EBC[111,112].

Metabolomics studies can be particularly informative when interpreted in conjunction with other ‘omics evaluations. For example, one of the largest gene wide association studies to date in CF identified associations between disease severity and expression of the gene APIP[113], which encodes an enzyme involved in the methionine salvage pathway, and metabolites associated with this pathway, including polyamines and free adenine, are associated with neutrophilic inflammation in CF[107,108,114]. Similarly, the lysophosphatidic acid receptor LPAR6 has been linked to CF lung disease in genomic studies[115], while the lysophospholipid substrates of this receptor are elevated in CF bronchitis[107,116]. While such studies demonstrate the promise of metabolomics to identify biomarkers and therapeutic targets, further study is needed before the potential of these identified pathways is truly known.

Conclusions

The intense inflammation present in the airways of individuals with CF is one of the most significant causes of progressive lung disease. Until we have a cure for CF, development of effective anti-inflammatories needs to be a priority for the CF research community. New approaches using metabolomics and other strategies to map the inflammatory targets in CF hold promise in development of new therapies.

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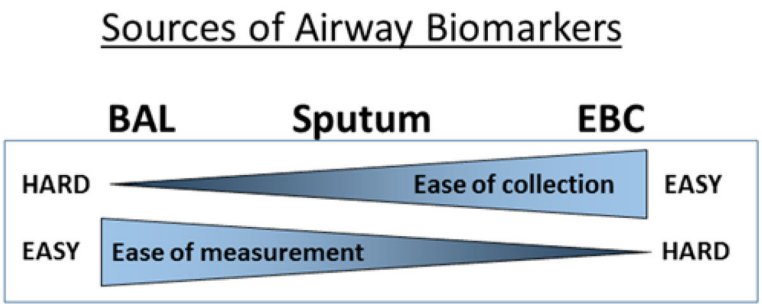

Figure 1.
Factors that contribute to the excessive inflammatory response in CF

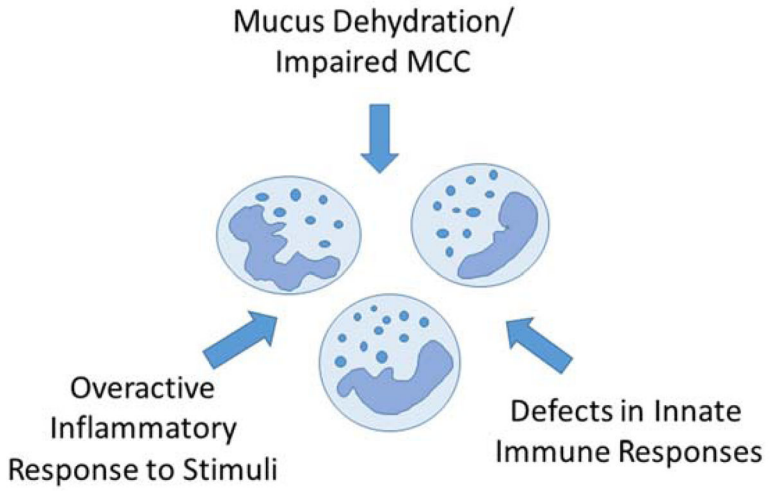

Figure 2.
Ease of collection and ease of biomarker measurement (including processing steps) are generally inversely related.

Table 1

Ideal inflammatory pathways to target

1. Involved in early disease
2. Associated with a biomarker that is readily measured in a non-invasive sample
3. Can be altered by a relatively simple pharmaceutical
4. Inhibition reduces inflammation without limiting ability to contain infection

Table 2

Comparison of airway samples for biomarker measurement

	Advantages	**Disadvantages**	**Region sampled**	**Inflammatory biomarkers**
Sputum	Well established Non-invasive	Requires immediate processing Difficult in young children	Most affected large airways	Cell counts Cytokines/proteins Metabolites
BAL	Can be used in all subjects	Higher risk (anesthesia) Expensive	Targeted smaller airways	Cell counts Cytokines/proteins Metabolites
EBC	Simple to collect Non-invasive	Very low and variable biomarker concentrations	Small airways (may under-sample plugged airways)	Cytokines/proteins Metabolites