Pilot study of inflammatory biomarkers in matched induced sputum and bronchoalveolar lavage from 2-year-olds with cystic fibrosis

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Abstract

Background: In this pilot study, we investigated whether induced sputum (IS) could serve as a viable alternative to bronchoalveolar lavage (BAL) and yield robust inflammatory biomarkers in toddlers with cystic fibrosis (CF) featuring minimal structural lung disease. Methods: We collected IS, BAL (right middle lobe and lingula) and blood, and performed chest computed tomography (CT) scans from 2-year-olds with CF (N=11), all within a single visit. Inflammatory biomarkers included 20 soluble immune mediators and neutrophil elastase (NE), as well as frequency and phenotype of T cells, monocytes / macrophages and neutrophils. Results: At the molecular level, nine mediators showed similar levels in IS and BAL (CXCL1, CXCL8, IL-1?, IL-1RA, IL-6, CCL2, CXCL10, M-CSF, VEGF-A), four were higher in IS than in BAL (CXCL5, IL-1?, CXCL11, TNFSF10) and two were present in IS but undetectable in BAL (IL-10, IFN-?). Meanwhile, soluble NE had lower activity in IS than in BAL. At the cellular level, T-cell frequency was lower in IS than in BAL. Monocytes / macrophages were dominant in IS and BAL with similar frequencies but differing expression of CD16 (lower in IS), CD115 and surface-associated NE (higher in IS). Neutrophil frequency and phenotype did not differ between IS and BAL. Conclusions: IS collected from 2-year-olds with CF yields biomarkers of early airway inflammation with good agreement with BAL notably with regards to molecular and cellular outcomes related to neutrophils and monocytes/macrophages.

INTRODUCTION

Cystic Fibrosis (CF) is the most frequent fatal genetic disease among Caucasian populations, with over 80,000 patients worldwide [1]. CF is an autosomal recessive disorder caused by one of more than 2,000 variants in the gene encoding the CF transmembrane conductance regulator (CFTR) anion channel. As such, newborn screening is now being widely implemented to diagnose children shortly after birth [2]. While multiple organs are affected in patients with CF, the majority of morbidity and mortality is due to airway disease [3]. Mutations in the CFTR protein, which is mainly expressed in epithelial cells, disrupts ion flow, resulting in early and sustained airway inflammation and mucus abnormalities [4]. This environment is also highly susceptible to chronic infection by opportunistic pathogens [5].

While recently approved CFTR-targeted modulator therapies are effective at restoring function of mutant channels, the control of ongoing inflammation remains a challenge [6]. Therefore, understanding and tracking early events in CF airway inflammation is essential for improving patient outcomes. In prior studies, extracellular neutrophil elastase (NE) activity measured in bronchoalveolar lavage (BAL) fluid at 3 months of age was found to be more effective than infection status and mucus obstruction at predicting lung damage at 1 and 3 years of age [7, 8]. Most of the extracellular NE activity in CF airway fluid stems from the active exocytosis of NE-rich granules by neutrophils acquiring the GRIM (granule releasing, immunomodu-

latory, and metabolically active) phenotype upon recruitment to CF airways [9, 10]. Live GRIM neutrophils are identifiable in BAL of young CF children and correlate positively with structural lung damage [11], as quantified by chest computed tomography (CT) scans [12].

While BAL is the gold standard for assessing CF airway inflammation [13], it is an invasive procedure that requires sedation and bronchoscopy, which often limits its use to clinically indicated procedures [14]. To better understand early events in CF airway inflammation, more frequent longitudinal sampling of young children using minimally invasive techniques would be desirable. One such option is induced sputum (IS), which has been used successfully to collect airway samples from children [15] and adults [9, 16] with CF. An important difference is that BAL primarily samples material from distal airways, while IS samples material from both distal and proximal airways. Recently, a study assessing the ability of IS to yield a relevant picture of lower airway microbiology in young children and adolescents with CF found similarities to BAL, but also notable differences [17]. With regard to inflammation, IS in adults with CF has shown similarities to BAL based on immune mediator levels, total leukocyte count and subset frequencies [16]. Critically, IS has not yet been used to study molecular and cellular biomarkers of inflammation in early CF lung disease.

To address this gap, we conducted a cross-sectional, single-center study of 2-year-olds with CF with sequential IS, BAL, and blood collections at the same visit. At this age, most patients have minimal structural lung damage, yet some have already developed signs of inflammation [18]. For BAL, we collected samples from the right middle lobe (RML) as a consistent measurement, and a separate sample collected from the lingula (LIN) [19, 20]. Via the analysis of soluble immune mediators and NE, leukocyte subsets and phenotype, and assessment of structural damage by chest CT scans, we demonstrate that IS can be used to detect early CF airway inflammation and lung damage, and document similarities and discrepancies with BAL fractions. Some of these results have been previously reported in the form of an abstract [21].

METHODS

Human subjects and samples. Data were collected from 11 2-year-old children at stable clinic visits. Subjects with CF were enrolled in the IMPEDE-CF study at Emory University and Children's Healthcare of Atlanta (Atlanta, GA, USA). All aspects of subject enrollment and sample collection were approved by the Emory University Institutional Review Board (IRB00097352). Consent for sample collection from subjects was obtained from parents on the day of the clinic visit. Subject demographics are summarized in **Table 1** . Collection of IS, blood, chest CT scan and BAL from the RML and LIN, were completed in that order within the same 4-hour visit. Samples were stored on ice following collection and delivered to the laboratory for immediate processing. Details of sample collection procedures are provided in online supplement and **Figure S1** , with sample yields indicated in **Table S1** .

Chest CT imaging and PRAGMA scoring. Chest CT scans were obtained under general anesthesia at Children's Healthcare of Atlanta [22] with full inspiratory and expiratory breath hold maneuvers, and scored using the validated PRAGMA-CF method [23]. Scoring of mucus plugging (%MP), bronchiectasis (%Bx) and abnormal airways (%AA) after excluding areas of atelectasis were done with inspiratory scans, and scoring of trapped air (%TA) used expiratory scans. Total Disease (%Dis) score was calculated by combining all abnormal areas as a percentage of total scored CT sections after excluding areas of atelectasis.

Total protein quantification. Total protein levels were quantified in IS and BAL using the Pierce copper sulfate/bicinchoninic acid (BCA) assay (ThermoFisher), with bovine serum albumin used for calibration.

Soluble immune mediator quantification. A custom assortment of 20 soluble immune mediators was measured in IS and BAL using a highly sensitive chemiluminescent assay (U-PLEX; Meso Scale Diagnostics), according to the manufacturer's protocol. To enable comparison between IS and BAL samples, mediator concentrations were normalized to total protein concentration (measured using BCA assay), as illustrated in **Figures 1 and 2** .

Flow cytometry. Multicolor flow cytometry was performed on cells from blood, BAL, and IS using previously described methods [11]. A gating strategy was developed to identify all major leukocyte populations in blood

and airway fluids, as detailed in**Figure S4** . Additional experimental details are presented in Supplemental Methods. Consistent fluorescence output was achieved through stringent cytometer calibration techniques, as detailed before [24].

Extracellular NE activity. Extracellular NE activities in IS, RML BAL and LIN BAL were measured with a Förster resonance energy transfer (FRET) assay using the NEmo-1 probe (Sirius Fine Chemicals SiChem GmbH), as previously described [25-27]. NE concentration was normalized to total protein concentration (measured using BCA assay) in the fluid.

Statistical analysis. Data were analyzed in Prism (GraphPad, version 8) using nonparametric statistics due to the limited number of samples, including the Wilcoxon matched-pairs signed rank test and Spearman test for correlation. Specifically for cytokines, a value of half the lower limit of detection or twice the upper limit of detection was assigned for data points which fell outside the limits of detection. Those values are clearly labeled as closed symbols in related figures. To avoid over-representation of imputed values, statistical comparisons of mediator concentration were not performed where more than half the data points of a sample group were imputed values. To conduct correlations, imputed values were removed, and correlations were conducted using R only for mediators with at least 6 non-imputed data points.

RESULTS

Parallel collection of IS, BAL and blood, and chest CT imaging in 2-year-olds with CF. The ability to collect IS from young children with CF was shown in prior studies but focused on a limited set of cytokines [15] and on microbiology [17]. To assess the potential of IS to yield data on an extended set of inflammatory biomarkers, we collected it in parallel to BAL from the RML and LIN, blood and chest CT, all within a single study visit. We attempted and achieved collection of all samples and data on 11 subjects (demographics in**Table 1**), except for two subjects from whom we were unable to obtain IS, thus lowering our analyzable set of IS samples to 9 (**Table S1**). Chest CT scoring confirmed that this cohort was at an early stage of airway disease, with the total disease score (%Dis) ranging from 0 to 5.9% (**Table S2**).

Similarities and differences in soluble immune mediators in IS and BAL. Prior studies of BAL have shown that onset of neutrophilic inflammation is a key determinant of structural lung damage in young children with CF [18]. Thus, we selected 11 mediators impacting neutrophil recruitment and activation (**Figure 1**). Five of those mediators showed similar levels in IS and BAL, namely CXCL1, CXCL8 (IL-8), IL-1α, IL-1RA, and IL-6. Six of those mediators showed differential levels between IS and BAL: CXCL5, G-CSF, IL-1β, and TNF-α were higher in IS than in one or both of the BAL fractions while IL-18 was lower in IS than in LIN BAL. Finally, IL-10 was below the limit of detection in most BAL samples but was measurable in most IS samples. We also assessed 9 mediators impacting recruitment and activation of monocytes/macrophages and T cells (**Figure 2**). Four of those mediators showed similar levels in IS and BAL, namely CCL2, CXCL10, M-CSF, and VEGF-A. Two of those mediators, CXCL11 and TNFSF10, showed higher levels in IS than in BAL.Finally, CCL4 and IFN-γ were below detection levels in most RML BAL samples while GM-CSF was below detection limit in most IS samples.

Correlations in soluble immune mediator levels among collected fractions. To explore potential relationships between the three airway fractions collected, we assessed correlations between IS and RML BAL, IS and LIN BAL, and RML and LIN BAL in the 20 immune mediators measured (**Figure S2**). Six neutrophil-associated mediators had significant positive correlations between IS and one or both of the BAL fractions, namely, CXCL8, G-CSF , IL1α, IL-1RA, IL-6 and TNF-α. Two monocyte/macrophage-associated mediators had significant positive correlations between IS and one or both of the BAL fractions, namely, M-CSF and VEGF-A. Between RML and LIN BAL fractions, mediators with significant positive correlations included four neutrophil-associated mediators (CXCL5, CXCL8, IL-1β, and IL-6) and three monocyte/macrophage -associated mediators (CXCL11, GM-CSF and VEGF-A).

Next, we assessed cross-correlations of the three airway fluid fractions with plasma (**Figure S3**). In all three airway samples, we observed positive correlations between CXCL8 and IL-1β and CXCL11 with TNFSF10. In IS and RML BAL, TNF-α and TNFSF10 correlated positively and G-CSF and IL-1RA correlated negatively.

In IS and LIN BAL, CCL2 and IL-6 correlated positively. In RML and LIN BAL, CXCL11 correlated positively with CCL2, CXCL10, IL-1β and TNF-α and IL-1RA correlated positively with TNF-α. Plasma cross-correlations differed from those in airway samples except for positive correlations between IL-10 and IL-18 observed in both plasma and IS; IL-1RA and TNF-α observed in plasma and both BAL fractions; and CXCL8 and IL-1α as well as IL-1RA with IL-18 observed in both plasma and RML BAL.

Cellular analysis of IS yields monocyte/macrophage, T cell, and neutrophil subsets, with phenotypes similar to BAL. By flow cytometry, we gated live monocytes/macrophages, T cells and neutrophils in BAL as we showed before [11], and successfully applied the same gating strategy to IS (**Figure S4**). Relative frequencies of neutrophils and monocytes/macrophages were similar in IS and BAL fractions, with a predominance of monocytes/macrophages in both. In contrast, the frequency of T cells was significantly lower in IS compared to BAL (**Figure 3A**). Neutrophils were activated in IS compared to blood based on increased surface expression of CD66b (secondary granule exocytosis marker). However, surface CD63 (primary granule exocytosis marker) was not significantly increased in IS compared to blood, likely due to the very early stage of airway disease in this 2-year-old cohort. CD16 expression was reduced on BAL, but not IS, neutrophils compared to blood (**Figure 3B**). Monocytes/macrophages in IS had significantly higher surface levels of CD115 (M-CSF receptor), while surface expression of CD163 (scavenger receptor) trended lower, as compared to BAL. Monocytes/macrophages in IS also showed decreased surface expression of CD16, which is sensitive to NE-mediated cleavage (**Figure 3C**).

We further analyzed neutrophil populations by determining the proportion of neutrophils demonstrating the GRIM phenotype ($CD63^{high}$ $CD16^{low}$, as shown in**Figure S4**). The prevalence of these highly exocytic neutrophils was not significantly different between IS and BAL. Although there was substantial dynamic range among airway samples, all blood samples had <1% GRIM neutrophils. Despite the high prevalence of GRIM neutrophils in some IS samples, soluble NE (following release from the primary granules of neutrophils) was lower in IS than in BAL (**Figure 4A**). Extracellular NE can be scavenged by neutrophils and monocytes/macrophages, which we assessed by surface staining with flow cytometry. In contrast to the difference with soluble NE measurement, surface NE on neutrophils and monocytes/macrophages did not differ between IS and BAL, while blood cells had lower surface NE, as expected (**Figure 4B**).

DISCUSSION

Based on the analysis of select soluble immune mediators as well as leukocyte subset frequency and phenotype, findings of this pilot study suggest that collection of IS in 2-year-olds with CF is achievable and yield inflammatory biomarkers that are broadly comparable to BAL. As highly effective CFTR modulator therapy is expected to be introduced earlier and earlier in the course of early CF airway disease in coming years, ongoing changes are expected in the start and rate of progression of structural damage and development of bronchiectasis. This further emphasizes the need for identifying and validating non-invasive markers of lung disease that can be used to monitor the presence of lower airway inflammation in young children with CF. It is also important to recognize the regional heterogeneity that occurs in the initial stages of CF lung disease and performing BAL in one bronchopulmonary segment of a lobe may not accurately represent the changes that are occurring in other lung segments. IS has the advantage that it represents an aggregate sample that is reflective of global changes while likely overrepresenting diseased regions (with ongoing mucus impaction). In addition, IS is not affected by the dilution effect of serial lavages that are performed during the collection of a BAL sample. It is important to recognize intrinsic differences between IS and BAL as we investigate inflammatory markers best suited for disease monitoring via each sampling technique.

Out of the 20 soluble immune mediators selected for analysis, GM-CSF was the only one below the detection limit in the majority of IS samples, and IL-18 was the only one with lower levels in IS than in BAL. The other 18 were at equal or greater abundance in IS compared to BAL. This may be explained by the convergence of secretions from distal airways (reflected in BAL) to large airways (reflected in IS) and will benefit IS-based studies by enabling detection of mediators that may be below detection limits in BAL. Importantly, this pilot study shows that established markers of early inflammation in CF airways are measured robustly in IS. Among these, CXCL8 (IL-8) is one of the most potent neutrophil chemoattractants in CF lung disease

[28], along with IL-6 and TNF-α. VEGF-A contributes to vascular permeability [29] and IL-1RA serves as a counter mechanism to IL-1α- and IL-1β-mediated pro-inflammatory signaling [30]. IL-1α itself was comparable between IS and BAL and is one of the first pro-inflammatory mediators present in early lung disease [31], which makes its measurement particularly critical.

By flow cytometry, IS neutrophils exhibited significant exocytosis of the secondary granules compared to visit-matched blood cells, reflecting a similar shift from blood as for BAL neutrophils. We previously described significant exocytosis of neutrophil primary granules in early CF lung disease, as evidenced by high CD63 and low CD16 expression characteristic of CF airway GRIM neutrophils [11]. Here, we observed similarly high proportion of GRIM neutrophils in IS and BAL, demonstrating that this key functional shift in neutrophils can be captured from distinct regions of the airways. As a result of primary granule exocytosis, NE is released into the extracellular environment, which we and others have shown to be associated with symptoms and severity of lung disease [7, 11]. Measurements of surface and soluble NE demonstrated some differences between IS and BAL in this study. Surface NE trended lower on IS than BAL neutrophils but was greater on IS monocytes/macrophages. In addition, soluble NE activity in IS was significantly reduced in IS compared to BAL. Taken together, these findings suggest that NE may be differentially compartmentalized in IS and BAL and that although sufficient NE is present in the IS to be captured on the surface of scavenger cells, its activity in IS may be inhibited by the antiprotease shield, as we previously suggested [11].

Besides phenotypic data of specific cell populations, comparing the presence of each major leukocyte class in the airways can provide important insights on status of lung disease. Early in disease, the CF airway lumen is dominated by monocytes/macrophages with occasional T cells. As disease progresses, neutrophils infiltrate the lumen with T cells largely excluded (reviewed in [32]). As expected in this 2-year-old cohort, monocytes/macrophages were dominant in BAL, and present in equal proportions in IS samples. Neutrophils were present to a lesser degree, which is also typical of early CF airway disease, and were equally represented in BAL and IS samples, too. T cells, however, while representing between 1-10% of total live leukocytes in BAL samples, were present at less than 1% in all IS samples. Thus, T cell numbers appear to dwindle more precociously in IS than in BAL at the onset of CF airway disease, a finding that warrants validation in a larger, longitudinal cohort of patients.

There are limitations to this pilot study. First, IS collection from young children requires additional maneuvers (use of high frequency chest wall oscillation and pharyngeal suctioning) and additional infection control precautions for the caregiver (see details in Supplemental Methods). Another limitation lies in the limited volume and cell yield inherent to IS collection in young children. This highlights the importance of applying sample-sparing/multiplexed and highly sensitive assays like the ones used in this study to the analysis of IS. Of note, in order to conduct side-by-side comparison of IS to BAL, we chose to normalize the calculated concentrations of soluble mediators and NE to that of total proteins. This method is likely a better approach than normalizing to volume, considering the possible differences in absorption of saline into the airway tissue for BAL, the variable output per lavage attempt, and the expected differences in density of IS samples. However, we previously noted total protein in BAL increases with PRAGMA-%Dis score [11, 33], likely as leukocytes secrete proteins into the lumen. As a result, protein normalization may also normalize in part to inflammatory burden. Finally, an important limitation of IS collection is the possibility for contamination of the sample by other fluids, including saliva and nasal passage secretions. In this study, we took particular precautions during the collection procedure to minimize such contamination (see Supplemental Methods for details).

In sum, the concomitant recruitment of neutrophils and acquisition of the GRIM phenotype, disappearance of T cells, and cytokine profile showing both pro- and anti-inflammatory mediators signifies that these patients who have minimal signs of lung damage by chest CT scan may still presen with measurable immunological signatures of progressing towards chronic CF lung disease. In this pilot study, we demonstrated that IS can be employed successfully in young children with CF to collect valuable data for multiparameter analysis of early CF lung disease. These findings warrant further testing of IS into disease monitoring efforts and interventional trials in early CF.

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Author contributions

Conceptualization: RT, LG; Data curation: VDG, DMG, GLS, LG; Formal analysis: VDG, DMG, LP, RT; Funding acquisition: RT, LG; Investigation: VDG, CM, MRB; Methodology: RT, LG; Project administration: RT, LG; Supervision: RT, LG; Validation: LP, RT, LG; Visualization: VDG, DMG, RT; Writing – original draft: VSG, RT; Writing – review & editing: VDG, DMG, CM, MRB, GLS, JDC, LP, RT, LG.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

Data sharing. All data used to produce graphs for soluble immune mediators, NE, and leukocyte phenotype and frequency are available for public access [34] at the following URL: https://data.mendeley.com/datasets/dsyp24db7h/2

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