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Neutrophils from F508del cystic fibrosis patients produce IL-17A and express IL-23 - dependent IL-17RC

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ABSTRACT

Cystic fibrosis (CF) is a chronic pulmonary disease that is associated with persistent microbial infection and chronic neutrophil infiltration, and also with elevated production of the pro-inflammatory cytokine IL-17A (IL-17). In the current study, we examined IL-17 and the inducible IL-17RC receptor subunit in neutrophils from *Pseudomonas aeruginosa* infected F508del CF patients at the time of pulmonary exacerbation, and again following intravenous antibiotic treatment. Neutrophils expressed *Il17a* and *Il17rc* transcripts and protein at the time of pulmonary exacerbation, which were absent following antibiotic treatment. Further, CF sputum induced IL-23 – dependent *Il17rc* expression in neutrophils from healthy individuals. Similarly, IL-17 producing neutrophils were detected in F508del and *Cftr*$^{-/-}$ mice infected intranasally with *P. aeruginosa*. In the sputum of CF subjects, the percentage IL-17 producing neutrophils correlated with elastase and MMP9 activity; therefore, this population of neutrophils may be an important contributor to the severity of pulmonary disease in CF patients.

1. Introduction

Cystic fibrosis (CF) is caused by mutations in a chloride channel known as the cystic fibrosis transmembrane conductance regulator (CFTR). Although over 2000 mutations have been reported, the absence of phenylalanine at position 508 (Phe508del, F508del) accounts for over 60% of the mutations in North American (1 in ~3000 births) and European Caucasians [1]. The F508del is a class II loss of function mutation, and impaired chloride efflux in pulmonary ciliated epithelial cells results in reduced water content in the airway surface liquid, and defective mucociliary clearance [1]. Formation of plaques within hypoxic niches enables *P. aeruginosa* to transition to a mucoid phenotype, which is more resistant to antibiotics and is associated with chronic infection [2,3], although a recent study demonstrates that the *P. aeruginosa* phenotype depends on their location in the lungs [4].

The bacterial infection combined with CFTR dysfunction in pulmonary epithelial cells results in significantly increased production of pro-inflammatory and chemotactic cytokines including IL-8, which is associated with neutrophil infiltration to infected lungs [5–8]. CF epithelial cells also produce increased GM-CSF, which contributes to the longevity and persistence of neutrophils in the lungs. However, CF neutrophils also have an intrinsic defect in their ability to phagocytose and kill *P. aeruginosa* [3], and the persistence of bacteria contributes to the long-term presence of neutrophils. Activation of neutrophils in the CF lung stimulates secretion of elastase and matrix metalloproteinases (MMP) including MMP9 that are known to contribute to irreversible damage to the airway wall and loss of pulmonary function [9,10].

Kolls et al. reported high levels of IL-17A, IL-17F and IL-23 in the sputum of CF subjects undergoing pulmonary exacerbation, which decreased following antibiotic therapy [11]. These investigators also showed elevated IL-17A in bronchoalveolar lavage samples from a pediatric population of CF patients [5]. Further, Tan et al., and Brodlie et al. used an immunohistochemical approach to examine biopsy specimens and explanted lungs, and showed multiple cell sources of IL-17, including CD4 cells, γδT cells, invariant natural killer (iNKT) cells and neutrophils [12,13]. Kolls and colleagues also identified Th17 memory cells in lymph nodes and parenchymas of post-transplant lungs [14].

Given the large number of infiltrating neutrophils in CF lungs, and that we reported *Il17a* gene expression in murine and human

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neutrophils [15–17], we used quantitative PCR, flow cytometry and fluorescence microscopy to examine neutrophils isolated from the sputum and peripheral blood of CF subjects before and after antibiotic treatment. Our findings clearly demonstrate that neutrophils in the sputum and blood of F508del CF subjects at the time of pulmonary exacerbation express IL-17 RNA and protein. These cells also express the inducible IL-17RC subunit of the receptor, and are associated with elevated elastase and MMP9 activity compared with post-treatment neutrophils. IL-17 production by neutrophils may therefore contribute to tissue damage in the lungs of patients with CF.

2. Materials & methods

2.1. Subject cohort

Eleven adult subjects (age 21–47 years) with CF who were admitted to Rainbow Babies and Children's Hospital for treatment of a pulmonary exacerbation were recruited for participation in this study (Table 1). CF participants carried at least one copy of F508del, and had chronic *P. aeruginosa* lung infection that required treatment with IV antibiotics. Five subjects were also infected with methicillin resistant *Staphylococcus aureus* (MRSA), and one subject was also infected with *Aspergillus fumigatus*. Pulmonary exacerbation was defined by the managing physician, who also determined the IV antibiotic treatment regimen. All subjects were able to spontaneously expectorate sputum. Blood and sputum were obtained within 2 days of admission and again 2 weeks after the initiation of IV antibiotics. One subject received a second 2-week course of antibiotics. All studies were approved by the University Hospitals Case Medical Center Institutional Review Board, and were registered on Clinicaltrials.gov (NCT02025829). Written informed consent was received from participants prior to inclusion in the study.

2.2. Sputum processing

Samples of spontaneously expectorated sputum were obtained from each subject at admission and after approximately 2 weeks of intravenous antibiotic therapy for treatment of a pulmonary exacerbation. Specimens were transported on ice. 0.1% of dithiothreitol (10% Sputolysin; Calbiochem-Novabiochem Corp., San Diego, CA) was used to homogenize the sputum. The specimen was incubated in a 37 °C water bath for 15 min and then centrifuged at 250*g* for 10 min at 4 °C. Supernatants were removed from the cell pellet and centrifuged at 4000*g* for 20 min at 4 °C. Cell pellets were used for cellular studies. After the second centrifugation, supernatants were divided into two aliquots. One aliquot was treated with the protease inhibitors, phenylmethylsulfonylfluoride (Sigma Diagnostics, St. Louis, MO) and ethylenediaminetetraacetic acid (Sigma Diagnostics), and the other aliquot was not treated with protease inhibitors. Supernatants were frozen at −80 °C for subsequent analysis of inflammatory mediators. Free neutrophil elastase and MMP9 activity was measured in the untreated supernatants. Other inflammatory mediators were measured in supernatants treated with protease inhibitors.

Table 1
Characteristics of study population.

Subject ID	Age	Sex	Genotype	Antibiotic Treatment	Admit Culture[a]
101	47	F	G551D/F508	Cetazidime, Colistimethate	*P. aeruginosa*
103	29	M	621 + 1G- > T/F508	Cetazidime, Colistimethate, Ceftaroline	PA, MRSA
104	28	F	F508/F508	Ceftaroline, Colistimethate	PA, MRSA
105	33	M	F508/F508	Ceftazidime, Colistimethate	PA, MSSA
106	33	M	F508/F508	Aztreonam, Ceftazidime	*P. aeruginosa*
107	32	M	1898 + 1G- > A/F508	Ceftazidime, Meropenem	PA, *Aspergillus fumigatus*
108	42	F	F508/F508	Meropenem, Colistimethate	*P. aeruginosa*
109	26	M	F508/F508	Meropenem, Colistimethate, Linezolid	PA, MRSA
110	26	M	F508/F508	Cetazidime, Colistimethate	*P. aeruginosa*
111	21	F	F508/N1303 K	Aztreonam, Colistimethate, Vancomycin	*P. aeruginosa*
112	24	F	F508/F508	Meropenem, Colistimethate, Linezolid	PA, MRSA

[a] *Pseudomonas aeruginosa* (PA) was recovered from all patients; MRSA: methicillin resistant *Staphylococcus aureus;* MSSA: methicillin sensitive *S.* aureus.

2.3. Neutrophil isolation

Neutrophils were isolated from sputum by negative selection using magnetic beads, and peripheral blood neutrophils were isolated by Ficoll density gradient centrifugation. Murine neutrophils were isolated from the bronchoalveolar lavage by negative bead selection. In all cases, neutrophil purity was assessed by Wright-Giemsa staining, and was generally >95%. Magnetic bead isolations used the EasySep human or mouse neutrophil enrichment kit (Stem Cell Technologies, Vancouver Canada).

2.4. MMP9 and neutrophil elastase activity assays

Cells were separated from the sputum by centrifugation, and supernatants were diluted to meet the parameters for detection. MMP9 was detected through activation of a modified pro-detection peptide and cleavage of the chromogenic peptide substrate (S-2444). Neutrophil elastase was detected using an elastase fluorescent substrate, which is cleaved by active elastase. In both assays, proteases were quantified by spectrophotometry at excitation/emission wavelengths 485/525 nm. Data were expressed as pg/ml according to manufacturer's instructions (MMP9 Biotrak Activity Assay, GE Healthcare; Neutrophil Elastase Activity Assay, Caymen Chemical).

Following magnetic bead purification, human neutrophil purity was confirmed by flow cytometry using antibodies to CD66 (clone CD66a-B1.1-anti-human CD66, eBioscience). To detect intracellular IL-17A, neutrophils were fixed, permeabilized, and incubated with antibodies to human IL-17A.

In mice, bronchoalveolar lavage from each animal was collected in PBS, and total cells were recovered following centrifugation, and suspended in 100 μl PBS. Neutrophils were incubated with the Ly6G monoclonal antibody NIMP-R14, which we found specifically identifies neutrophils [17], and the total number of neutrophils per mouse was quantified by flow cytometry. For intracellular IL-17A, neutrophils were pooled from BAL from five mice, fixed and permeabilized, and the percent IL-17 + ve neutrophils was calculated in 30,000 events.

2.5. Confocal imaging

Images were collected using a Leica DMI6000B microscope equipped with HCX PL APO 100× / 1.4 oil immersion objective and an attached UltraVIEW VoX spinning disk confocal system (Perkin Elmer, Waltham, MA). Metamorph Image Analysis Software (Molecular Devices Corp., Downington, PA) was used to examine images as described [18].

2.5.1. Quantitative PCR

RNA was extracted from all neutrophils, and analyzed for *IL17a* and *Il-17rc* expression by qPCR as described previously [18]. *GAPDH* (human) *or Actb* (mouse) were used as loading gel controls. PCR products were qualitatively analyzed on 2% agarose gel. Quantitative CT scores are shown in Supplemental Figure 1.

2.5.2. Murine P. aeruginosa CF models

Cftr$^{tm1Kth\text{-}del\ F508}$ (delF508), and *Cftr*^{tm1Unc} (Cftr$^{-/-}$) mice were obtained from the CF Animal Infection and Inflammation Modeling Core,