MAJOR ARTICLE

Inflammatory Responses to Individual Microorganisms in the Lungs of Children With Cystic Fibrosis

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Background. We hypothesized that the inflammatory response in the lungs of children with cystic fibrosis (CF) would vary with the type of infecting organism, being greatest with *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Methods. A microbiological surveillance program based on annual bronchoalveolar lavage (BAL) collected fluid for culture and assessment of inflammation was conducted. Primary analyses compared inflammation in samples that grew a single organism with uninfected samples in cross-sectional and longitudinal analyses.

Results. Results were available for 653 samples from 215 children with CF aged 24 days to 7 years. A single agent was associated with pulmonary infection ($\geq 10^5$ cfu/mL) in 67 BAL samples, with *P. aeruginosa* ($n = 25$), *S. aureus* ($n = 17$), and *Aspergillus* species ($n = 19$) being the most common. These microorganisms were associated with increased levels of inflammation, with *P. aeruginosa* being the most proinflammatory. Mixed oral flora (MOF) alone was isolated from 165 BAL samples from 112 patients, with 97 of these samples having a bacterial density $\geq 10^5$ cfu/mL, and was associated with increased pulmonary inflammation ($P < .001$). For patients with current, but not past, infections there was an association with a greater inflammatory response, compared with those who were never infected ($P < .05$). However, previous infection with *S. aureus* was associated with a greater inflammatory response in subsequent BAL.

Conclusions. Pulmonary infection with *P. aeruginosa*, *S. aureus*, or *Aspergillus* species and growth of MOF was associated with significant inflammatory responses in young children with CF. Our data support the use of specific surveillance and eradication programs for these organisms. The inflammatory response to MOF requires additional investigation.

The lungs of children with cystic fibrosis (CF) become infected progressively throughout childhood with a variety of microorganisms, including *Staphylococcus aureus*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* [1–7]. The introduction of bronchoalveolar lavage (BAL) programs into CF clinics in the 1990s led to the recognition that inflammation and lower airway infection may be present very early in life even in the absence of overt respiratory signs and symptoms [1, 2, 8, 9]. Inflammation is present in the lungs of many infants with CF with cell counts exceeding those in healthy infants [9, 10] and increased levels of inflammatory cytokines, eg, interleukin 8 (IL-8); neutrophil enzymes, including myeloperoxidase and neutrophil elastase (NE); and markers of oxidative stress [1, 7–9, 11, 12].

Despite the postulated role of pulmonary infection in the initiation and/or maintenance of pulmonary

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inflammation [2, 11], the contribution of individual pathogens to the inflammatory response has not been well documented. Lower respiratory infection with *P. aeruginosa* is associated with higher levels of IL-8, a major neutrophil chemoattractant [1, 2, 13–15], and free NE activity [16]. Coculture of *P. aeruginosa* and *S. aureus* from the lungs may lead to an even greater inflammatory response in children, suggesting a synergistic effect of multiple organisms on pulmonary inflammation [15]. The pathogenicity of certain microorganisms cultured from the lower respiratory tract in children, such as *H. influenzae*, *Escherichia coli*, and *Candida* and *Aspergillus* species, remains unclear [17] and warrants additional investigation.

The present study was undertaken to examine the inflammatory response associated with organisms commonly found in the lungs of children with CF, using both cross-sectional and longitudinal analyses. We hypothesized that not all of these agents would induce the same inflammatory response and that *P. aeruginosa* and *S. aureus* would be the most proinflammatory.

METHODS

Participants

The early surveillance program run by the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) is a clinical pulmonary microbiology surveillance program, in which children up to the age of 7 years undergo an annual assessment that includes a chest computed tomography scan, bronchoscopy, and BAL for determination of pulmonary infection and inflammation, conducted in the CF clinics at Princess Margaret Hospital for Children (PMH), Perth, and the Royal Children's Hospital (RCH), Melbourne, Australia. The diagnosis of CF in children is made by means of a sweat test [18] following detection by newborn screening, or after presentation with clinical symptoms suggestive of CF. The full methodology has been published previously [9, 19], and further details are available from the AREST CF website (http://www.arestcf.org).

Two hundred fifteen children with CF who participated in the surveillance program during the period from January 1999 through November 2009 were included in the present report. Demographic characteristics are shown in Table 1. Clinical management was in accordance with the standard protocols in use in each clinic. Antistaphylococcal prophylaxis with amoxicillin clavulanate is recommended for children up to the age of 2 years. Children in whom *P. aeruginosa* is detected in the BAL fluid are entered into an eradication program [16]. Parents gave specific consent for BAL fluid to be used for research purposes, especially measurement of inflammation in the lungs. The surveillance program and this specific study were approved by the Ethics Committees of PMH and RCH and conform to the guidelines for conduct of research in children from the National Health and Medical Research Council, Australia.

Table 1. Demographic Characteristics of Individual Children Included in the Study

Characteristic	No. of samples
No. of children	215
No. of visits	653
Center	
Melbourne	75 (35)
Perth	140 (65)
Age at diagnosis, median days (range)	28 (0–1848)
Diagnosis	
Following newborn screening	142 (66)
Family history	10 (5)
Failure to thrive	11 (5)
Meconium ileus	19 (8)
Respiratory symptoms	10 (5)
Combination presentation	8 (4)
Other	15 (7)
Symptoms at diagnosis	
None	69 (32)
Gastrointestinal	75 (35)
Respiratory	20 (9)
Combination	27 (13)
Not reported	24 (11)
Sex	
Male	112 (52)
Female	103 (48)
Genotype	
Phe508del homozygous	114 (53)
Phe508del heterozygous	83 (39)
Other	7 (3)
Pancreatic insufficiency[a]	171 (80)

NOTE. Data are no. (%) of patients unless otherwise specified.

[a] Low fecal elastase activity.

Bronchoalveolar Lavage

Bronchoscopy and BAL were performed under general anesthesia, using a total intravenous protocol, at a time when the children were considered to be clinically stable and not suffering from pulmonary exacerbation, as previously reported [8, 9]. Suction of pulmonary secretions was delayed until the tip of the bronchoscope was below the level of the carina to avoid upper airway contamination. Three aliquots of normal saline (1 mL/kg body weight) were instilled into the right middle or lower lobe and retrieved using low-pressure suction [20]. The first aliquot was sent to the respective Department of Microbiology for culture and the identification of bacteria, fungal elements, and viruses. The remaining 2 aliquots were stored on ice until pooled and processed (within 3 hours of collection) for assessment of inflammation.

Microbiology

BAL was cultured on blood, cysteine lactose electrolyte-deficient agar, Fildes agar, and Sabouraud agar with chloramphenicol.

Viruses were detected by using direct immunofluorescence and culture. By convention, a bacterial density of $\geq 10^5$ colony-forming units (cfu)/mL was taken to indicate pulmonary infection. However, to allow a full examination of the inflammatory response to individual organisms, bacterial densities from 10^2 to 10^8 cfu/mL were analyzed. Samples from which a variety of organisms were cultured, but with no one type predominant, were classified as mixed oral flora (MOF). To overcome the potential limitation of false-negative BAL culture results, the children who had never had an organism cultured from their BAL fluid (never infected) were used as the control group. Molecular diagnostic techniques were not available.

Cytology

Total and differential cell counts were performed as previously reported [8, 9].

Inflammation

IL-8 was measured in BAL fluid supernatant using an enzyme-linked immunosorbent assay (BD Opt EIA, BD Biosciences) with a working range of 10–6400 pg/mL as previously reported [8]. Free NE activity was measured using an activity assay [21], with a working range of 0.02–12.0 μg/mL [8, 9].

Statistical Analysis

The effects of each pathogen on inflammatory response were estimated using linear mixed effects models, with random effects for age to take into account the within-subject correlation. Response variables for BAL were log-transformed, because all were skewed. An inflammatory response score was created by taking a weighted average of the logged BAL responses: log total cell count/mL fluid retrieved, log neutrophils/mL fluid retrieved, log NE, and log IL-8. The weights were derived using principal component analysis. Summary statistics of clinical and inflammatory characteristics were calculated using multiple assessments per participant. Linear mixed models, with random effects for age, were also used to assess the association between infection status at time t and inflammatory response at time $t + 1$. The models were adjusted for the confounding effects of age, pancreatic insufficiency, detection by newborn screening, and presence of respiratory symptoms. Analyses were carried out using STATA, version 10.1 (StataCorp).

RESULTS

A total of 653 BAL samples were obtained from 215 children during the study period; a single BAL samplel was obtained from 45 children, 2 BAL samples from 58 children, 3 BAL samples from 41 children, 4 BAL samples from 27 children, and ≥5 BAL samples from 43 children. No microorganisms were cultured from 213 BAL samples, and of this group, 101 came from children who were "never infected." A single microorganism was isolated, at any density, from 128 BAL samples. *S. aureus* ($n = 31$) and *P. aeruginosa* ($n = 29$) were the 2 most frequently isolated single pathogens. MOF alone was isolated from 165 BAL samples from 112 patients, with 97 of these samples having a bacterial density $\geq 10^5$ cfu/mL (Table 2). A single organism was causally associated with pulmonary infection ($\geq 10^5$ cfu/mL) in 67 BAL samples, with *P. aeruginosa* ($n = 25$) and *S. aureus* ($n = 17$) the most common (Table 2). More than 1 microorganism was isolated from 148 BAL samples, with *S. aureus*, *H. influenzae,* and *P. aeruginosa* being the most common (Table 2). In 50 BAL samples, MOF was reported in conjunction with a predominant infecting organism (Table 2).

A greater inflammatory response was seen in BAL samples from which single or multiple organisms were isolated, compared with the never infected control group: higher group median neutrophil counts (multiple organisms $P < .001$), higher levels of free NE activity ($P < .001$), and higher levels of IL-8 ($P < .001$) (Table 3). The level of inflammation in BAL samples that came from children with infection detected in the past, but not currently infected, did not differ from those from children who were never infected (Table 3).

The degree of inflammation associated with individual microorganisms is shown in Table 4. These raw data show higher group median levels of total cells, neutrophils, NE activity, and IL-8 in children infected with *P. aeruginosa* or *S. aureus*.

The "inflammatory response score" was used as the outcome variable to examine the effect of individual microorganisms on pulmonary inflammation (Table 5). Neither genotype ($P = .74$), antistaphylococcal prophylaxis ($P = .84$), nor sex ($P = .50$) contributed significantly to the overall model. However, pancreatic insufficiency ($P = .01$), detection by newborn screening ($P = .03$), age ($P < .001$), and respiratory symptoms ($P < .001$) contributed significantly and were therefore included as covariates in estimating adjusted values to make comparisons between various groupings of infection. This analysis confirmed that there were no differences in inflammatory response in children who had "never" been infected, compared with those who were currently uninfected but had experienced an infection ≥1 year ago (coefficient, 0.08; standard error [SE], 0.20; $P = .70$). The greatest inflammatory responses were seen with *P. aeruginosa*, *S. aureus*, and *Streptococcus pneumoniae;* with smaller but significant inflammatory responses associated with *Aspergillus* species and *H. influenzae*. A significant inflammatory response was also associated with MOF (Table 5). BAL samples that grew multiple organisms were associated with a greater inflammatory response score, compared with those from children who were never infected (coefficient, 0.58; SE, 0.14; $P < .001$). The inflammatory response to a coinfection with *P. aeruginosa* and *S. aureus* was significantly greater than in BAL samples from children who

Table 2. Microorganisms Isolated From 653 Bronchoalveolar Lavage Samples at Any Density and at ≥10^5 cfu/mL (Bacteria Only)

Microorganism	Single organism		Multiple organisms[a]	
	Any density	≥ 10^5 cfu/mL[b]	Any density	≥ 10^5 cfu/mL[b]
Bacteria				
Mixed oral flora	165	97	50[c]	46[c]
Achromobacter species	0	0	1	0
Burkholderia cepacia	0	0	1	1
Escherichia coli	8	6	3	3
Enterobacter species	4	2	3	3
Haemophilus influenzae	12	8	34	31
Klebsiella species	0	0	1	0
Moraxella species	4	1	2	1
Pseudomonas aeruginosa	29	25	32	29
Serratia species	0	0	2	2
Staphylococcus aureus	31	17	54	45
Staphylococcus epidermidis	1	1	0	0
Stenotrophomonas maltophilia	2	2	7	5
Streptococcus pneumoniae	6	5	5	4
Streptococcus pyogenes	0	0	2	2
Group C streptococcus	0	0	2	2
Fungi				
Aspergillus species	19	…	13	…
Candida species	7	…	12	…
Scedosporium species	0	…	1	…
Viruses		…		…
Adenovirus	0	…	1	…
Cytomegalovirus	1	…	1	…
Parainfluenza virus	1	…	0	…
Respiratory syncytial virus	2	…	2	…

[a] Multiple organisms refer to samples from which >1 organism was isolated.

[b] Organisms listed where they are part of a multiple infection and where their bacterial density is ≥10^5 cfu/mL.

[c] Data are mixed oral flora isolated together with a predominant pathogen.

were never infected ($P < .001$), and the inflammatory response score for combined infections was greater than for infection with either organism alone; however, possibly as a result of the small number of combined infections, this difference did not reach statistical significance ($P = .14$).

The influence of bacterial density on the inflammatory response is shown in Table 5. BAL samples that grew ≥10^5 cfu/mL of *Aspergillus* species (including *Aspergillus niger* and *Aspergillus fumigatus*), *P. aeruginosa, S. aureus,* or *S. pneumoniae* were associated with a significantly greater inflammatory response ($P < .001$). Smaller but significant inflammatory responses were also seen in BAL samples that grew ≥10^5 cfu/mL of *H. influenzae, E. coli,* or MOF. The inflammatory response associated with an infection with *Candida* species or *Enterobacter* species was not significantly greater than that in children who were classified as never infected. With the exception of *S. aureus* ($n = 14$) and MOF ($n = 68$), there were too few samples to separately examine the inflammatory response associated with bacterial densities of <10^5 cfu/mL. Although there seemed to be a greater inflammatory response with higher bacterial density for *S. aureus* (regression coefficient, 0.68 ± 0.26 with <10^5 cfu/mL to 1.14 ± 0.23 with ≥10^5 cfu/mL), this difference did not reach statistical significance ($P = .08$). Bacterial density did not seem to influence inflammatory response for MOF, with coefficients of 0.39 ± 0.14 and 0.46 ± 0.12, respectively ($P = .82$).

Longitudinal analyses were conducted to determine the influence of previous infection status on current BAL inflammatory status. Previous infection with *S. aureus* (any bacterial density) was associated with an increased inflammatory response score on the subsequent BAL ($n = 31$; regression coefficient, 0.55 [95% confidence interval {CI}, .05–1.05]; $P = .03$). This was not seen with previous infection with *P. aeruginosa* ($n = 29$; regression coefficient, 0.14 [95% CI, −0.36 to 0.65]; $P = .58$), infection with organisms other than *S. aureus* or *P. aeruginosa* ($n = 60$; regression coefficient, 0.16 [95% CI, −.21 to .54]; $P = .38$), or with infection with organisms other than *S. aureus* or *P. aeruginosa* or growth of MOF ($n = 225$; regression coefficient, 0.17 [95% CI, −.11 to .46]; $P = .24$).

Table 3. Clinical and Inflammatory Characteristics Associated With Current Bronchoalveolar Lavage (BAL) Infection Status

Clinical characteristics	No current infection (BAL $n = 213$)	Past infection[a] (BAL $n = 112$)	Never infected[a] (BAL $n = 101$)	Single organism[b] (BAL $n = 292$)	Multiple organisms[b] (BAL $n = 148$)
Age at which BAL sample collected, mean years (95% CI)	2.56 (2.29–2.83)	3.46 (3.10–3.81)	1.45 (1.06–1.83)	2.46 (2.23–2.70)	2.54 (2.23–2.86)
No. of unique children	129[c]	69	65	151	101
Sex (male:female)[d]	65:64	36:33	32:33	75:76	58:43
Genotype (homozygous Phe508del), %[e]	55.7	55.1	60.0	52.0	56.3
Diagnosis following newborn screening, proportion (%)[e]	109/129 (84.5)	59/69 (85.5)	54/55 (83.1)	116/151 (76.8)	72/101 (71.3)
Pancreatic insufficiency, proportion (%)[e]	96/120 (80.0)	59/68 (86.8)	42/57 (73.7)	122/144 (84.7)	89/100 (89.0)
Inflammatory markers					
Total cell count, $\times 10^3$ cells/mL fluid retrieved[e]	254 (218–295)	260 (212–320)	242 (194–301)	296 (258–338) $P = .13$	408 (342–487)[f] $P = .001$
Neutrophil count, $\times 10^3$ cells/mL fluid retrieved[e]	33 (26–43)	28 (20–40)	33 (22–47)	207 (165–260)[f] $P < .001$	181 (132–248)[f] $P < .001$
Neutrophil elastase, ng/mL[e]	155 (126–190)	159 (120–210)	144 (106–195)	239 (198–287)[f] $P = .001$	346 (272–441)[f] $P < .001$
Interleukin-8, pg/mL[e]	404 (324–503)	470 (348–636)	322 (234–444)	672 (552–819)[f] $P < .001$	856 (660–1112)[f] $P < .001$
Inflammatory response score, mean (95% CI)	4.36 (4.22–4.49)	4.36 (4.17–4.55)	4.28 (4.08–4.48)	4.97 (4.84–5.10)[f] $P < .001$	5.12 (4.96–5.29)[f] $P < .001$

NOTE. CI, confidence interval; BAL, bronchoalveolar lavage.

[a] Mutually exclusive subsets of "no current infection" group.

[b] Infection defined as bacterial density is $\geq 10^5$ cfu/mL, excluding mixed oral flora.

[c] 6 children are included in both the never infected and past infected groups.

[d] Characteristics of the unique children.

[e] Geometric mean (95% CI).

[f] $P < .05$ compared with "never infected." Means were estimated using linear mixed models with random subject effects. Individual children may be included in >1 group. Inflammatory response score was calculated by using a principal component analysis to create a single summary measure of the logged BAL responses: log total cell count/mL fluid retrieved; log neutrophil count/mL fluid retrieved; log NE; and log IL-8. Weights for each component were obtained, averaged, and used to create a summary measure called "inflammatory response score."

DISCUSSION

The results from this study present a novel assessment of the inflammatory response to pulmonary infection with single organisms commonly found in the lungs of young children with CF. Although the number of children infected with a single organism was relatively small, those infected with *P. aeruginosa*, *S. aureus*, and *Aspergillus* species had significantly higher levels of neutrophilic inflammation and proinflammatory cytokines than did uninfected children. In contrast, the inflammatory

Table 4. Individual Components of the Inflammatory Profile of Bronchoalveolar Lavage Fluid From Children Who Were Never Infected or From Whom a Single Organism or Mixed Oral Flora Was Isolated

Organism	Total cell count, $\times 10^3$ cells/mL fluid retrieved	Neutrophil count, $\times 10^3$ cells/mL fluid retrieved	Neutrophil elastase, ng/mL	Interleukin-8, pg/mL
Never infected ($n = 101$)	242 (194–301)	33 (22–47)	144 (106–195)	322 (234–444)
Aspergillus species ($n = 19$)	272 (166–446)	329 (161–675)	552 (334–914)	687 (385–1226)
Candida species ($n = 7$)	183 (87–388)	203 (72–572)	368 (171–791)	679 (258–1788)
Escherichia coli ($n = 8$)	275 (133–572)	294 (104–837)	100 (52–192)	387 (147–1017)
Haemophilus influenzae ($n = 12$)	278 (155–498)	69 (31–156)	445 (244–811)	1138 (551–2352)
Mixed oral flora ($n = 165$)	264 (224–310)	223 (173–286)	142 (123–164)	499 (395–630)
Pseudomonas aeruginosa ($n = 29$)	493 (315–770)	187 (101–349)	1124 (696–1815)	2467 (1464–4157)
Staphylococcus aureus ($n = 31$)	509 (357–725)	155 (90–266)	736 (464–1168)	1016 (620–1664)

NOTE. Data are geometric mean (95% confidence interval), estimated using linear mixed models with random subject effects. Statistics were not performed on individual components of the inflammatory response, and the data are presented here for completeness only.

Table 5. Inflammatory Responses to Individual Organisms

	Any bacterial density				Bacterial density $\geq 10^5$ cfu/mL			
Organism	*n*	Coefficient	SEM	*P*	*n*	Coefficient	SEM	*P*
Single organism								
Aspergillus species	19	0.89	0.24	<.001	7	1.19	0.33	<.001
Candida species	7	0.46	0.32	.15	0	…	…	…
Escherichia coli	8	0.35	0.34	.30	6	0.64	0.33	.05
Enterobacter species	4	0.43	0.43	.31	2	…	…	…
Haemophilus influenzae	12	0.68	0.27	.01	8	0.59	0.31	.05
Moraxella species	4	−0.05	0.45	.90	1	…	…	…
Pseudomonas aeruginosa	29	1.07	0.24	<.001	25	1.32	0.22	<.001
Staphylococcus aureus	31	1.05	0.20	<.001	17	1.12	0.23	<.001
Streptococcus pneumoniae	6	1.38	0.37	<.001	5	1.31	0.38	<.001
Mixed oral flora	165	0.42	0.11	<.001	97	0.51	0.12	<.001
Multiple organisms	148	0.58	0.14	<.001	…	…	…	…

NOTE. Regression analyses, adjusted for pancreatic insufficiency, detection at newborn screening, age, and the presence of respiratory symptoms, comparing the inflammatory response score associated with the presence of individual organisms in BAL fluid samples compared with the inflammatory response score associated with BAL fluid samples from children in the never infected group. SEM, standard error of the mean.

response to other agents, including *Candida* species and *E. coli,* was less pronounced, not differing from that of the never infected group. A significant inflammatory response was also seen to MOF grown from BAL fluid.

Before discussing the implications of our findings, a number of issues require examination. The extent of inflammation in BAL increases with age regardless of the presence of infection [16]. In addition, as has been reported elsewhere, the prevalence of infection with *P. aeruginosa* increases with age and may follow initial infection with other microorganisms [7, 22, 23]. Also, our inflammatory outcomes (total cell count, number of neutrophils, IL-8 levels, and free NE activity) were highly autocorrelated. We attempted to circumvent these problems by using a principal component analysis and then using the results of this to construct an equally weighted "inflammatory response score." The primary analyses were performed by comparing the inflammatory response scores from BAL samples collected from children who were never infected with those from samples containing $\geq 10^5$ cfu/mL single or multiple microorganisms. The grouped data in Table 3 need to be treated with caution, because they do not allow for the potential confounding of both inflammation and infection by age. However, the analyses presented in Table 5 have been corrected for age and show that the greater inflammatory responses cannot be accounted for simply by the age of the child.

The small numbers of BAL samples that were culture-positive for *H. influenzae, E. coli,* and other less commonly isolated gram-negative bacteria necessitate caution when interpreting the associated inflammatory response in BAL fluid. Thus, although these data suggest that these organisms are associated with either no greater or only modestly elevated inflammatory responses, compared with those observed in children "never infected," we cannot draw more definite conclusions regarding their pathogenicity. In a similar light, the increased inflammatory responses seen with *S. pneumoniae* are based on only 6 BAL samples. The BAL samples analyzed in the present study were collected at a time when the children were clinically stable and fit for elective general anesthesia. Thus, we identified very few respiratory viruses (Table 2) and were unable to examine associated inflammatory responses. In addition, we have relied on conventional microbiological techniques and not used molecular diagnostic techniques. We may, therefore, have missed low-level infections and misclassified children as being uninfected. However, this potential error would tend to minimize the differences between groups and cannot explain the results presented.

The data from the present study demonstrating a relatively greater inflammatory response in patients infected with *P. aeruginosa* and *S. aureus* are consistent with the recent report from Sagel et al [15], who reported increased inflammation and more clinical disease in patients infected with *P. aeruginosa* and *S. aureus*. Where our data differ from those reported by Sagel et al [15] is that although there was a trend to a greater inflammatory response in inflammation in patients coinfected with both *P. aeruginosa* and *S. aureus* when compared with infection with either alone, this did not reach statistical significance. There are a number of possible explanations for these differences. Although it is not possible from the report of Sagel et al [15] to determine the number of children infected with *S. aureus*, they had more children infected with *P. aeruginosa* ($n = 59$) than we did ($n = 25$); thus, increased study power is certainly one possibility. We also use an aggressive eradication program for *P. aeruginosa* [16], and chronic infection is uncommon in young children in our clinics.

The strong inflammatory response associated with isolation of *Aspergillus* species from BAL fluid in the present study was not expected. In the absence of symptoms and laboratory test results consistent with allergic bronchopulmonary aspergillosis, a report of *Aspergillus* species in BAL or sputum samples is unlikely to lead to specific antifungal treatment. Although our data do not prove that these fungi cause problems, the fact that they are associated with an inflammatory response indicates that additional studies to examine the clinical importance of isolating *Aspergillus* from the lungs are warranted.

A large number of the BAL samples in the present study grew MOF ($n = 165$), including 97 in which the bacterial density was $\geq 10^5$ cfu/mL. We took considerable care to avoid contaminating the BAL with upper airway secretions; BAL was performed through a laryngeal mask airway that isolated the upper airway, and suction was avoided until the bronchoscope had passed the carina. However, we cannot completely exclude the possibility that some upper airway contamination occurred. Cultures of MOF from lower airways are generally regarded as benign. However, a recent study examining the oral flora of healthy infants [24] revealed a wide variety of bacteria, including *S. aureus, P. aeruginosa* (nonmucoid), *H. influenzae*, *E. coli,* and *Moraxella (Branhamella) catarrhalis,* thus demonstrating that potentially pathogenic bacteria may be present in children's upper airways. The increased inflammatory response score associated with MOF, both at any bacterial density and at $\geq 10^5$cfu/mL (Tables 4 and 5), is concerning and suggests that further analysis of what constitutes MOF is warranted.

There is an increasing realization that the lungs are not sterile but have a resident bacterial flora [25–27]. Unbiased metagenomic approaches show differences in the pulmonary microbiome in asthmatics compared with controls, in both children and adults [25, 26]. Few data exist on the resident pulmonary microbiome in infants and young children with CF; however there is a suggestion of a change in the pulmonary microbiome with age [27]. These data raise the possibility that selection pressures, either intrinsic to the CF lung or induced by treatments given, result in an alteration of the pulmonary microbiome over age. In the present study, the inflammatory response to MOF is heavily age dependent, with MOF being more commonly grown in BAL samples from older children and being associated with greater inflammation in older children as well. One potential explanation is that the organisms that comprise MOF in BAL fluid change with age and/or with antibiotic use, with more proinflammatory organisms becoming more common at older ages. These possibilities warrant further investigation.

In summary, the data from the present study reveal significant inflammatory responses to *P. aeruginosa*, *S. aureus,* and *Aspergillus* species. Specific eradication protocols are available and effective for *P. aeruginosa,* and our data suggest that similar protocols directed against *S. aureus* are worth investigating. The role of *Aspergillus* species and MOF in pulmonary infection of children with CF requires further examination.

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