Innate Lung Defenses and Compromised *Pseudomonas aeruginosa* Clearance in the Malnourished Mouse Model of Respiratory Infections in Cystic Fibrosis

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Cystic fibrosis (CF) is characterized by dysfunction of the digestive and respiratory tracts resulting in generalized malnutrition and chronic respiratory infections. Chronic lung infections with *Pseudomonas aeruginosa*, intense neutrophil-dominated airway inflammation, and progressive lung disease are the major cause of high morbidity and mortality in CF. Here we investigated the effects of malnutrition in CF on innate lung defenses, susceptibility to *P. aeruginosa* colonization, and associated inflammation, using aerosol models of acute and chronic infections in normal, malnourished, and transgenic mice. *CFTR<sup>min1Unc−/−</sup>* knockout mice displayed body weight variations and showed variable pulmonary clearance of *P. aeruginosa*. This variability was not detected in bitransgenic *CFTR<sup>min1Unc−/− (FABP-hCFTR)* mice in which the intestinal defect had been corrected. Diet-induced protein calorie malnutrition in C57BL/6J mice resulted in impaired pulmonary clearance of *P. aeruginosa*. Tumor necrosis factor alpha (TNF-α) and nitrite levels detected upon exposure to *P. aeruginosa* aerosols were lower in the lungs of the malnourished C57BL/6J mice relative than in lungs of mice fed a normal diet. The role of TNF-α and reactive nitrogen intermediates in *P. aeruginosa* clearance was tested in TNF-α and inducible nitric oxide synthase (iNOS) knockout mice. *P. aeruginosa* clearance was diminished in transgenic TNF-α- and iNOS-deficient mice. In contrast to the effects of TNF-α and iNOS, gamma interferon knockout mice retained a full capacity to eliminate *P. aeruginosa* from the lung. Malnutrition also contributed to excessive inflammation in C57BL/6J mice upon chronic challenge with *P. aeruginosa*. The repeatedly infected malnourished host did not produce interleukin-10, a major anti-inflammatory cytokine absent or diminished in the bronchoalveolar fluids of CF patients. These results are consistent with a model in which defective *CFTR* in the intestinal tract leads to nutritional deficiency which in turn contributes to compromised innate lung defenses, bacterial colonization, and excessive inflammation in the CF respiratory tract.

Cystic fibrosis (CF), the most common inheritable lethal disease among Caucasians, is caused by mutations in the *CFTR* gene encoding a chloride channel (CF transmembrane conductance regulator [CFTR]) (10). CF is characterized by chronic obstructive pulmonary disease, intestinal problems, and generalized malnutrition (30). As the disease progresses, the lungs of CF patients become infected and colonized with a variety of pathogens. Among these, *Pseudomonas aeruginosa* represents the predominant CF pathogen (15). Persistent pulmonary infections with *P. aeruginosa*, intense neutrophil-dominated airway inflammation, and progressive lung disease are presently the major cause of high morbidity and mortality in CF (23).

Several concurrent proposals have been put forward to explain the relationship between the defect in *CFTR* and predilection for *P. aeruginosa* infections (4, 5, 12, 22, 31, 36, 40). The altered electrolyte composition of CF epithelial secretions has been linked to reduced bacterial properties of defensins (12, 36). CFTR has also been implicated in *P. aeruginosa* uptake by respiratory epithelial cells (31). Reduced sialylation of glycoconjugates on the surface of epithelial cells in CF has been associated with increased *P. aeruginosa* adhesion (40). Other studies focusing on cytokine profiles in bronchoalveolar lavage fluids of CF patients have suggested that the excessive inflammation in the CF lung may be attributed to endogenously increased levels of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL-8) (5, 22). Considering the multitude of sometimes conflicting models, factors rendering the CF lung prone to infections are still not conclusively defined.

Chronic malnutrition with progressive weight loss has been recognized as a problem in CF (30). Almost 50% of newly diagnosed CF infants suffer from various degrees of malnutrition (25), maldigestion, and malabsorption (21, 28). About 85% of CF patients show pancreatic insufficiency and severe steatorrhea due to the problems with their digestive systems (30). Approximately 16% of CF newborns and adults also present with meconium ileus or meconium ileus equivalent, one of the most common causes of intestinal obstruction in CF (7, 11). CF patients have increased energy expenditure and energy deficit and are underweight. The combined effects of malabsorption and anorexia in CF result in inefficient utilization of proteins, lipids, vitamins, and trace elements. Significant growth retardation with weight loss has been seen in all age groups of CF patients (30). The issues related to protein energy malnutrition (PEM) in CF are of particular significance due to their import on long-term prognosis in this disease (24).

Here we tested whether some aspects of *P. aeruginosa* infections in CF could be explained by effects of malnutrition on relevant innate defenses of the respiratory system. We used an aerosol infection model in combination with several strains of transgenic mice and normal mice subjected to PEM (which represents one aspect of nutritional problems in CF) to model the role of innate defenses in *P. aeruginosa* pulmonary clear-
ance. We report the roles of malnutrition, relevant proinflam-
matory and anti-inflammatory cytokines, and other mediators of
innate lung immunity in P. aeruginosa clearance from the
respiratory tract.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For nebulization, P. aeruginosa PA01 was grown in 200 ml of Luria broth at 37°C for 12 h and harvested by centrifugation at 6,000 x g for 15 min at 4°C. Pellets (1.2 [wet cell mass] were washed once in cold 1% Proteose Peptone (Difco) phosphate-buffered saline (pH 7.4) and resuspended in cold phosphate-buffered saline. The suspension was adjusted to 10^{11} CFU/ml and 5 ml was used for nebulization as previously described (39).

Animals. Mice were 8 weeks old at the inception of single- or repeated-aerosol-exposure experiments as previously described (39). All animals were housed under specific-pathogen-free conditions. C57BL/6j and transgenic mice (four per group) were from The Jackson Laboratory (Bar Harbor, Maine). Gamma interferon (IFN-γ) knockout mice were C37BL6-Ifnγ−/− (JR2287); inducible nitric oxide synthase 2 (iNOS-2) knockout mice were C57BL/6-Nos2−/− (JR2609). CFTR transgenic mice were CFTRm1UNC−/− mice purchased from The Jackson Laboratory and genotyping mice following instructions from the supplier. CFTRm1Unc−/− (FAPB-hCFTR) mice were purchased from The Jackson Laboratory and breeding colony maintained without further typing. CFTRm1Unc−/− (FAPB-hCFTR) mice have their intestinal defect corrected by the presence of a functional human CFTR gene expressed from a rat intestinal fatty acid-binding protein gene promoter (41).

Infection model. The aerosol infection model and equipment (Glas-Col, Terre Haute, Ind.) were as previously described (39). For single-exposure experiments, animals were sacrificed 18 h upon exposure to P. aeruginosa aerosols. For chronic infection model (39), animals were repeatedly subjected to P. aeruginosa aerosols every 72 h (eight times over a period of 22 days) and sacrificed 18 h following the last exposure. Bacteriological analysis and histopathology workup were as previously described (39). Relative bacterial survival represents the percent of CFU remaining in the lungs compared to the initially deposited CFU in the lungs determined by sacrificing a group of animals immediately following the aerosol exposure (39).

Generation of PEM in mice. A standard protocol was followed (38). Low (2%)- and high (20%)-protein and protein-free diets were from BIOSERV (Frenchtown, N.J.). These diets were made isocaloric to the basic diet (casein-based mouse diet) by carbohydrate supplement. The protein-free diet consisted of 7% fat, 5% fiber, 3% ash, and 79% carbohydrate. Compositions of low- and normal-protein diets, respectively, were as follows: protein (2% and 20%), fat (7% and 7%), fiber (5% and 5%), ash (3% and 3%), and carbohydrate (77% and 57%). The protein component in the high-protein diet was hydrolyzed casein. The caloric content of normal-protein diets was 21.6 kcal/g. Analysis of protein, fat, and carbohydrate content of diets was performed based mouse diet) by carbohydrate supplement. The protein-free diet consisted of low (PEM)- or normal (control)-protein diets was 2 weeks or after switching of low- to normal-protein diet for additional 2 weeks (PEM-recovered [PEM-]). Normal C57BL/6 mice in which PEM was induced showed an 11-fold-reduced capacity to clear P. aeruginosa aerosols. For chronic infection model (39), animals were repeatedly subjected to P. aeruginosa aerosols every 72 h (eight times over a period of 22 days) and sacrificed 18 h following the last exposure. Bacteriological analysis and histopathology workup were as previously described (39). Relative bacterial survival represents the percent of CFU remaining in the lungs compared to the initially deposited CFU in the lungs determined by sacrificing a group of animals immediately following the aerosol exposure (39).

Respiratory clearance in CFTR transgenic mice with and without correction of the intestinal defect: effects of malnutrition. Using an aerosol infection model (39), we observed variability among CF knockout mice (37) in the ability to clear P. aeruginosa (Table 1). The CFTRm1Unc−/− mice presented with two extremes of either clearing or not clearing P. aeruginosa, a phenomenon not encountered with any other strains of age-matched mice in the mice used. This finding appeared to be associated with variations in body weight and nutritional status of the mice. In CFTRm1Unc−/− mice that had their intestinal defect corrected by the presence of a functional human CFTR gene expressed from a rat intestinal fatty acid-binding protein gene promoter CFTRm1Unc−/− (FAPB-hCFTR) mice (41), P. aeruginosa was efficiently cleared from the lung and the variability was no longer observed (Table 1). One interpretation of these observations is that repairing the CFTR defect in the intestinal tract improved lung defenses against P. aeruginosa. Normal C57BL/6j mice in which PEM was induced showed a 11-fold-reduced capacity to clear P. aeruginosa (Fig. 1A, PEM). This defect was promptly restored by placing the malnourished mice on a normal-protein (20%) diet (Fig. 1A, PEM-R). These results indicate that malnutrition impairs pulmonary clearance of P. aeruginosa.

Clearance of P. aeruginosa in IFN-γ, iNOS, and TNF-α transgenic mice. As mutations in CFTR have severe effects on the digestive system, the resulting nutritional defect may impair the innate defense systems in the lung. PEM can selectively compromise the immune system in the lung, with IFN-γ, TNF-α, and iNOS being the major effector mechanisms affected (9). To test whether and which of these factors play a role in P. aeruginosa clearance from the lung, we investigated the roles of IFN-γ, iNOS, and TNF-α in corresponding transgenic mice. Age-matched IFN-γ, iNOS, and TNF-α transgenic knockout and C57BL/6j mice were exposed to P. aeruginosa aerosols. The strongest effect on P. aeruginosa was observed in TNF-α transgenic mice, which showed a 19-fold decrease in the efficiency of P. aeruginosa clearance relative to C57BL/6j mice (P < 0.01). The iNOS knockout animals also showed a threefold-reduced clearance (P < 0.01), while IFN-γ animals displayed no defect in removal of P. aeruginosa from the lungs (P = 0.786). These results suggest that iNOS and TNF-α play important roles in the innate resistance to P. aeruginosa and its clearance from the respiratory tract.
Malnutrition affects iNOS and TNF-α output in response to respiratory infection with *P. aeruginosa*. Considering the significant role of iNOS and TNF-α, we next tested whether NO and TNF-α elicited by respiratory infection with *P. aeruginosa* were affected by malnutrition. TNF-α was reduced 2.8-fold ($P < 0.01$) (Fig. 2A) and NO production (determined by measuring levels of its metabolite nitrite) was reduced by 58% ($P < 0.01$) (Fig. 2B) in C57BL/6J PEM mice relative to controls fed a normal-protein diet. Reduced iNOS levels in CF epithelial cells have been independently noted (19). Histopathological examination indicated an increase in the neutrophil and other inflammatory cell infiltration in the lungs of mice under PEM (Fig. 3). The neutrophil-recruiting chemokines MIP-2 and KC, which represent functional equivalents of human IL-8 in the mouse (16, 34, 35), were increased (50% for MIP-2 [$P < 0.01$] and 60% for KC [$P < 0.05$]) in C57BL/6J PEM animals relative to mice fed normal diet (Fig. 2C). There was also a 30% increase in MPO activity in the PEM mice ($P < 0.05$) (Fig. 2D).

**Reduced levels of IL-10 in a chronically infected malnourished host.** In addition to increased levels of proinflammatory cytokines and neutrophil infiltration, another hallmark of CF is a low level of the major anti-inflammatory cytokine IL-10 in the bronchoalveolar fluid (5). In a model of chronic infection with *P. aeruginosa* that has been previously used to test effects of IL-10 on inflammatory processes due to *P. aeruginosa* infection (39), the clearance of *P. aeruginosa* remained less efficient in malnourished animals (Fig. 4A). We also observed production of significant amounts of IL-10 in the well-nourished mice 22 days following the initiation of a regimen of repeated exposure to *P. aeruginosa* aerosols (Fig. 4B). In contrast, the malnourished animals had no detectable IL-10 (Fig. 4B). In normally fed mice (which prior to infection showed less than 9.0 pg of IL-10 per g), IL-10 was present at a level of 86.5 ± 11.9 pg/g of lung tissue. IL-10 levels were below the detection limit (9.0 pg/g) in the malnourished animals (Fig. 4B). In the chronic infection model, PEM also resulted in a 2.1-fold increase in MIP-2 relative to animals on the normal-protein diet ($P < 0.05$; Fig. 4C) although the levels of KC were similar in both groups of mice ($P = 0.4679$). MPO levels in chronically infected PEM mice were 2.3-fold higher than in the normally fed mice ($P < 0.01$; Fig. 4C).

**DISCUSSION**

In this work, we report reduced clearance of *P. aeruginosa* from the respiratory tract of transgenic mice lacking TNF-α and iNOS, suggesting that these mediators of innate immunity may be critical for lung defenses against *Pseudomonas* infection. In contrast to TNF-α and iNOS transgenic mice, IFN-γ knockout mice do not show reduced *P. aeruginosa* clearance, suggesting that this cytokine does not play a major role in the early stages of *Pseudomonas* colonization. Any iNOS role in the control of *P. aeruginosa* immediately upon aerosol delivery is most likely independent of the IFN-γ-mediated induction of...
iNOS in murine phagocytic cells. The production of iNOS has been reported to be constitutive in both human and murine airway epithelia (19), and these cells could be the source of the effects observed in our model system. In vitro killing of *P. aeruginosa* with sodium nitroprusside (19), NO-dependent killing of *P. aeruginosa* in excised murine lungs (19), and reduced control of *P. aeruginosa* in animals treated with the iNOS inhibitor aminoguanidine (13) have also been reported. These analyses along with our findings using iNOS knockout animals are consistent with the conclusion that NO plays a role in *P. aeruginosa* control in the respiratory tract. Since iNOS levels and NO output are reduced in CF epithelia (19, 27), this deficiency may contribute to the colonization with *P. aeruginosa*.

The transgenic animals lacking TNF-α showed the most striking defect in clearing *P. aeruginosa* in our infection model. A role for TNF-α in the innate resistance to *P. aeruginosa* infection has also been proposed by others based on a correlation between TNF-α levels in BALB/c and C57BL/6 strains of mice and their differential susceptibility to endobronchial instillation of *P. aeruginosa* embedded in agar beads (13). Furthermore, depletion of TNF-α increases bacterial loads in some strains of mice (13), in keeping with our observations with transgenic animals. Buret et al. (8) have noticed that intratracheal administration of TNF-α in rats improves *P. aeruginosa* clearance from the lungs. The function of TNF-α in resistance to *P. aeruginosa* colonization most likely involves a number of mechanisms, including recruitment of neutrophils and macrophages via effects on adhesion molecules or chemoattractants or by affecting bactericidal activities of phagocytic cells. Our unpublished results indicate that bone marrow-derived macrophages from TNF-α knockout mice are diminished in the ability to kill *P. aeruginosa* during early time points, suggesting that this defect could be one of the contributing factors to the observed reduced *P. aeruginosa* clearance in TNF-α transgenic mice. Paradoxically, TNF-α levels are elevated in bronchoalveolar lavage fluids in CF (5), and yet the patients cannot clear *Pseudomonas* from their lungs. This dis-
crepancy can be best explained by the observation that the immunoreactive TNF-α in CF bronchoalveolar fluids is biologically inactive, as it is complexed with soluble TNF-α receptor which is also elevated in CF lung fluids (5). The TNF-α in CF is thus most likely not available to stimulate antipseudomonal activities in the respiratory tract.

In this report we also examined the effects of malnutrition on clearance and relevant cytokine profiles in the lung. A potential role for malnutrition in CF has been considered in a follow-up to the observation that variable body weight in CFTRm1Unc−/− mice with intestinal dysfunction was associated with variability in P. aeruginosa respiratory clearance. While we could not establish a statistically significant difference between CFTRm1Unc−/− and C57BL/6J mice, the variability in P. aeruginosa clearance was excessive in the CFTRm1Unc−/− group. Importantly, this variability was not observed in the bitransgenic mouse with the corrected CFTR defect in the intestinal tract. An observation suggesting increased susceptibility to P. aeruginosa infections in CF escalates with age, ranging from 1 to 25% in the age group from 0 to 4 years and rising to 80% in the age group from 10 to 14 and above (11, 29). The pattern of incidence of P. aeruginosa infections in CF, reaching a plateau between the age groups of 10 to 14 and 15 to 19 years, is often attributed to antibiotic treatment of infections caused by other pathogens (which conversely to P. aeruginosa decline in incidence with age), but additional or alternative explanations, potentially including effects of malnutrition, should not be excluded.

The results presented in this work indicate that malnutrition can compromise pulmonary defenses against P. aeruginosa col-

onization and is conducive to excessive inflammation in response to P. aeruginosa infection, resembling the situation in CF. PEM animals showed significantly reduced production of TNF-α and NO in response to P. aeruginosa challenge relative to the well-nourished experimental group. Importantly, neutrophil infiltration in the lungs of malnourished animals did not result in increased bacterial clearance from the lung and instead was a correlate of unproductive inflammatory response. PEM also prevented production of IL-10 during chronic infection with P. aeruginosa, the critical anti-inflammatory cytokine that lacking in the bronchoalveolar fluids in CF (5) and is necessary to control excessive inflammation in the murine lung chronically infected with P. aeruginosa (39).

In conclusion, we propose the following model: (i) defective CFTR in CF causes dysfunction of the digestive system, and (ii) the ensuing malnutrition adversely affects innate lung defenses contributing to bacterial colonization and associated inflammation in addition to other direct effects of CFTR in the lung (4, 5, 12, 22, 31, 36, 40). A prediction from the relationships uncovered in this work is that the function of CFTR in the intestinal tract may be as critical for lung defenses against P. aeruginosa, as its direct roles may be at the level of the respiratory epithelium (4, 5, 12, 22, 31, 36, 40). We propose that in order to improve the compromised innate immunity in the CF lung, future treatment efforts, including gene therapy and other means of correcting the CFTR defect, should consider potential benefits of similar interventions in the intestinal tract.

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REFERENCES


