

The role for neutrophil extracellular traps in cystic fibrosis autoimmunity

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While respiratory failure in cystic fibrosis (CF) frequently associates with chronic infection by *Pseudomonas aeruginosa*, no single factor predicts the extent of lung damage in CF. To elucidate other causes, we studied the autoantibody profile in CF and rheumatoid arthritis (RA) patients, given the similar association of airway inflammation and autoimmunity in RA. Even though we observed that bactericidal permeability-increasing protein (BPI), carbamylated proteins, and citrullinated proteins all localized to the neutrophil extracellular traps (NETs), which are implicated in the development of autoimmunity, our study demonstrates striking autoantibody specificity in CF. Particularly, CF patients developed anti-BPI autoantibodies but hardly any anti-citrullinated protein autoantibodies (ACPA). In contrast, ACPA-positive RA patients exhibited no reactivity with BPI. Interestingly, anti-carbamylated protein autoantibodies (ACarPA) were found in both cohorts but did not cross-react with BPI. Contrary to ACPA and ACarPA, anti-BPI autoantibodies recognized the BPI C-terminus in the absence of posttranslational modifications. In fact, we discovered that *P. aeruginosa*-mediated NET formation results in BPI cleavage by *P. aeruginosa* elastase, which suggests a novel mechanism in the development of autoimmunity to BPI. In accordance with this model, autoantibodies associated with presence of *P. aeruginosa* on sputum culture. Finally, our results provide a role for autoimmunity in CF disease severity, as autoantibody levels associate with diminished lung function.

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease in individuals of European descent (1, 2). Mutations in the CF transmembrane conductance regulator (CFTR) gene disrupt CFTR-controlled secretion of bicarbonate and chloride across airway epithelial cells (3). This impairment in ion transport across the epithelium enhances mucus viscosity and interferes with mucociliary clearance, thereby creating an environment susceptible to bacterial infection (4, 5). As a result, CF patients have recurrent respiratory bacterial infections, frequently involving *Pseudomonas aeruginosa*, which is detected in up to 85% of patients (6–8). Inevitably, persistent bacterial infection in CF patients leads to an influx of neutrophils into the lung that results in a state of chronic inflammation (9–12). Despite this characteristic immunopathology, CF patients demonstrate great clinical variability in their lung manifestations, which cannot be predicted by the nature of the *CFTR* mutation or the extent of bacterial infection (11–13).

Autoantibodies directed against bactericidal permeability-increasing protein (BPI) are found in CF patients and correlate with diminished lung function (14, 15). BPI (~55 kDa) is an antimicrobial peptide stored in azurophilic granules of neutrophils that is required for efficient clearance of gram-negative bacteria (16, 17). However, there is little understanding of the etiopathogenic role of this autoimmunity in CF. Studies of chronic inflammatory diseases, such as rheumatoid arthritis (RA), lupus, or granulomatosis with polyangiitis, have demonstrated a strong link between neutrophil-mediated inflammation and autoimmunity (18–20). Moreover, in RA, this inflammation is thought to initiate in the lung as a result of environmental factors, e.g., microbes or smoking (21). In particular, NETosis, a mechanism by which neutrophils extrude their DNA and protein contents to form neutrophil extracellular traps (NETs), is thought to lead to the breaking of tolerance to citrullinated and carbamylated proteins in RA patients (22). In this model,

Conflict of interest: The authors have declared that no conflict of interest exists.

Submitted: June 8, 2016

Accepted: September 15, 2016

Published: October 20, 2016

Reference information:

JCI Insight. 2016;1(17):e88912.

doi:10.1172/jci.insight.88912.

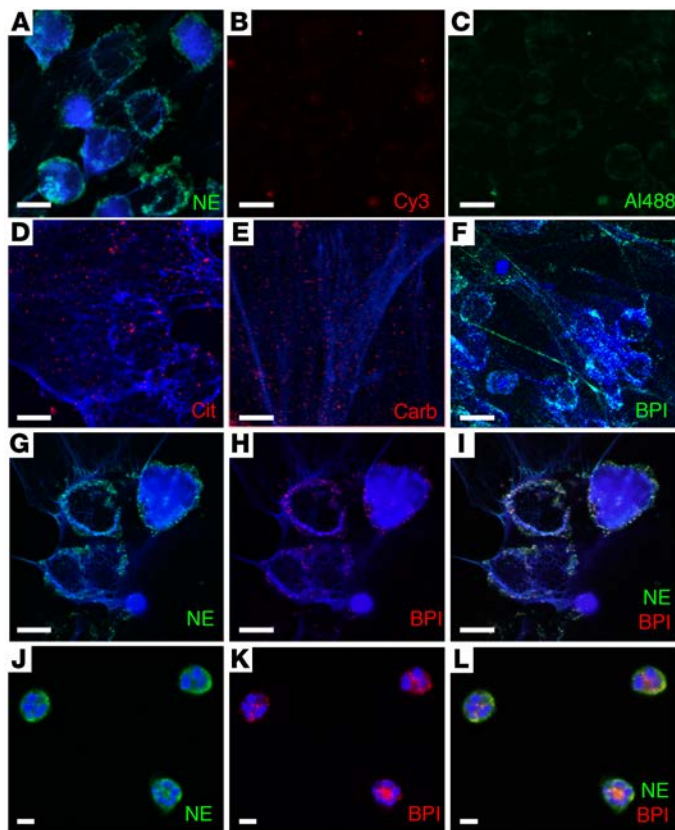


Figure 1. BPI and carbamylated proteins are localized on neutrophil extracellular traps. (A) PMA treatment (600 nM, 2 hours) of healthy neutrophils induced formation of neutrophil extracellular traps (NETs), characterized by extrusion of DNA (DAPI, blue) and neutrophil elastase (Al488, green). (B and C) Cy3 and Al488 alone were used to determine the level of nonspecific staining. Immunocytochemistry staining demonstrated that (D) citrullinated proteins (Cy3, red), (E) carbamylated proteins (Cy3, red), and (F) BPI-Al488 localize to the DNA strands of the NETs. (G–I) Neutrophil elastase (Al488) and BPI-Cy3 colocalize in the NETs. (J–L) Neutrophil elastase (Al488) and BPI-Cy3 colocalize in untreated PMNs. Scale bar: 10 μ m. NE, neutrophil elastase; BPI, bactericidal permeability-increasing protein; Cit, citrullinated proteins; Carb, carbamylated proteins.

neutrophil enzymes that localize to NETs induce posttranslational modifications, such as citrullination and carbamylation, thus creating neoantigens that lead to anti-citrullinated protein autoantibodies (ACPA) and anti-carbamylated protein autoantibodies (ACarPA) (22–26). Given the abundance of nucleic acids in NETs, the induction of autoantibodies by NETosis is likely to be facilitated by TLR 7/9-mediated B cell activation (27). While formation of NETs in the CF lung has been appreciated as both an antibacterial defense mechanism and a contributor to protease-induced lung damage (10, 28–30), the role of NETosis in CF autoimmunity has not been studied.

Even though pulmonary insufficiency remains the leading cause of morbidity and mortality in CF, the causes and manifestations of chronic airway inflammation appear to differ between CF patients (31–35). This interpatient variability could result from unique underlying CFTR defects, differences

in microbial infection, the associated immune responses, environmental influences, and disease-modifying genes (36, 37). Both the innate and adaptive immune systems shape the inflammatory environment of the CF lung and contribute to a complex and variable immunopathology that is not completely understood (38–40). In this article, we assess the nature of adaptive immunity in CF by comparing the autoantibody profile seen in adult CF and RA patients. As part of this study, we demonstrate the specificity of the anti-BPI immune response in CF and characterize its association with other known autoantibodies, bacterial infection, and lung function. Moreover, we propose the mechanism that leads to the breaking of tolerance to BPI in CF.

Results

BPI and other autoantibody targets in inflammatory diseases localize to the NETs. Following PMA-induced NET formation, we observed the expression of neutrophil elastase as well as citrullinated and carbamylated proteins on the decondensed DNA strands (Figure 1, A, D, and E). Moreover, BPI was found on the neutrophil membranes as well as on the NET DNA strands (Figure 1F). BPI frequently colocalized with neutrophil elastase, perhaps unsurprising given the dual release of these proteins from azurophilic granules (Figure 1, G–I, and J–L). Thus, BPI, like other autoantigens, is expressed in the context of extruded DNA in the NETs.

Autoantibody profile and specificity in CF patients. Given the presence of multiple autoantigens on NETs, we examined the possibility of overlapping autoantibody reactivity in CF ($n = 38$) and RA ($n = 50$). Autoantibodies targeting neutrophil-purified BPI (nBPI) were detected in 42% of CF serum samples, while none was detected in RA serum samples (Figure 2A and Table 1). ACPA-IgG and IgM rheumatoid factor were detected in less than 8% of 38 CF serum samples in our cohort and at very low levels compared with RA patients (Figure 2, B and C, and Table 1). These findings suggest that autoimmunity to BPI and citrullinated proteins develops via disease-specific mechanisms. In contrast, ACarPA were detected in 28% of RA serum samples and in 40% of CF serum samples (Figure 2D and Table 1). Thus, the presence of frequent ACarPA reactivity in CF and RA patients supports the notion of a shared pathway in the breaking of tolerance to this particular neoantigen.

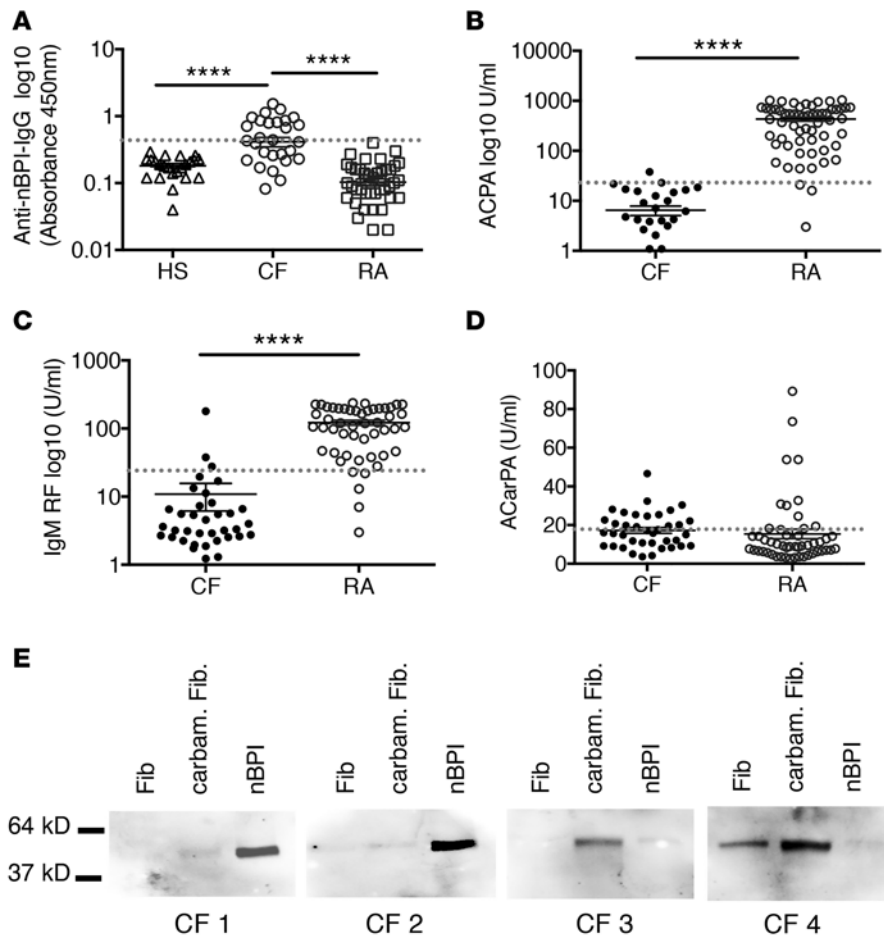


Figure 2. Autoantibody profile and specificity in CF patients. (A) Anti-nBPI IgG levels are higher in cystic fibrosis (CF) patients compared with those in healthy sera (HS) and rheumatoid arthritis (RA) patients by 1-way ANOVA, with Bonferroni post-hoc (HS [$n = 23$], mean = 0.15; CF [$n = 38$], mean = 0.41; RA [$n = 50$], mean = 0.10); dotted line represents positive cutoff determined as mean + 2 SD of HS cohort. (B) ACPA IgG levels are higher in RA patients compared with CF patients by t test (CF [$n = 38$], geometric mean [CI = 95%] = 6.47 U/ml; RA [$n = 50$], geometric mean [CI = 95%] = 430.5 U/ml); samples with ACPA levels >20 U/ml were considered to be ACPA positive. (C) IgM rheumatoid factor (RF) levels are higher in RA patients compared with CF patients by t test (CF [$n = 38$], geometric mean [CI = 95%] = 10.9 U/ml; RA [$n = 50$], geometric mean [CI = 95%] = 122 U/ml); samples from patients with IgM RF concentrations >25 U/ml were considered to be IgM RF positive. (D) ACPA IgG are present at comparable levels in CF and RA patients by t test (CF [$n = 38$], geometric mean [CI = 95%] = 17.2 U/ml; RA [$n = 50$], geometric mean [CI = 95%] = 15.4 U/ml); samples with ACPA concentrations >18 U/ml were considered to be ACPA positive (measured by Inova Diagnostics). (A–D) Error bars represent mean \pm SEM; **** $P < 0.0001$. (E) Reactivity of CF sera to denatured fibrinogen (Fib), carbamylated fibrinogen (carbam. Fib.), and nBPI (1 μ g) was evaluated by immunoblot. Representative CF sera bind to nBPI and carbamylated fibrinogen with specificity. ACPA, anti-citrullinated protein autoantibodies; ACPA, anti-carbamylated protein autoantibodies; nBPI, neutrophil-purified BPI.

Therefore, the relationship between anti-nBPI autoantibodies and ACPA was examined in CF sera. Surprisingly, no correlation between anti-nBPI autoantibodies and ACPA was found in CF patients by either immunoblotting (Figure 2E) or ELISA (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/jci.insight.88912DS1). Selected CF sera exhibited distinct patterns of reactivity with either nBPI or carbamylated fibrinogen by immunoblotting (Figure 2E). Moreover, several CF serum samples reacted with both fibrinogen and carbamylated fibrinogen but not with nBPI. Reactivity of CF sera to carbamylated fibrinogen parallels previously observed autoreactivity in RA patients (41). Thus, autoantibodies in CF sera react with specificity with the denatured forms of nBPI, fibrinogen, and carbamylated fibrinogen. Despite being coexpressed in some CF serum samples, anti-nBPI and ACPA lack cross-reactivity and appear to develop independently.

Autoantibody responses to BPI are directed at its C-terminus and do not require posttranslational modification. CF patient sera bound BPI in azurophilic granules of neutrophils in situ, thereby suggesting that anti-BPI autoantibodies do not detect posttranslationally modified neoantigens induced by NET formation. This was confirmed in several ways. First, no posttranslational modifications (carbamylation, citrullination, or isoaspartylation) of BPI were detected by proteomic analysis and isoAsp vapor assay (Supplemental Tables 1 and 2). Consistent with this finding, we did not detect colocalization between BPI and proteins containing either carbamylated or citrullinated neoepitopes by immunocytochemistry in PMA- or *P. aeruginosa*-induced NETs (Supplemental Figure 2). Second, using recombinant protein corresponding to the C-terminus of BPI (CTBPI, aa 227–487), we demonstrated a high level of correlation between IgG reactivity toward this protein fragment and that toward nBPI by ELISA and immunoblotting ($r = 0.69$, $P < 0.0001$) (Figure 3). Unsurprisingly, none of the RA sera reacted with CTBPI recombinant protein (data not shown). Thus, the reactivity of anti-BPI autoantibodies maps to CTBPI and derives from the primary amino acid sequence in the absence of any posttranslational modifications in the bacterially derived recombinant protein.

Table 1. Autoantibodies in chronic inflammatory diseases

	% Positive	
	CF sera (n = 38)	RA sera (n = 50)
ACPA ^A	5.3% (2/38)	94% (47/50)
IgM RF ^B	7.8% (3/38)	92% (46/50)
Anti-BPI IgG ^C	42% (16/38)	0% (0/50)
ACarPA ^D	40% (15/38)	28% (14/50)

^AACPA positive, ACPA concentration >20 U/ml. ^BIgM RF positive, IgM RF concentration >25 U/ml. ^CAnti-BPI IgG positive, anti-BPI IgG concentration >0.4 OD₄₅₀. ^DACarPA positive, ACPA concentration >18 U/ml. ACPA, anti-citrullinated protein autoantibodies; ACPA, anti-citrullinated protein autoantibodies; CF, cystic fibrosis; IgM RF, IgM rheumatoid factor; RA, rheumatoid arthritis.

C-terminus domains of BPI, we evaluated the role of *P. aeruginosa* elastase in BPI cleavage (43). Immunoblotting demonstrated that treatment of neutrophils with a *P. aeruginosa* elastase-specific knockout bacterial strain (*AlasB* PAO1) did not induce the appearance of the appropriately 30-kDa BPI fragment, in contrast to the wild-type PAO1 treatment (Figure 4C) (44). Similar results were obtained following treatment with *AlasR* PAO1 and *AlasR* PA14, additional elastase-deficient *P. aeruginosa* strains (Figure 4C). Variable BPI cleavage was observed in the untreated PMNs, suggesting an effect of neutrophil elastase (NT, Figure 4C). In the presence of elastase-deficient *P. aeruginosa*, this effect of neutrophil elastase appears attenuated, as BPI cleavage was only seen following treatment with elastase expressing *P. aeruginosa*. Therefore, our findings suggest that BPI cleavage in *P. aeruginosa*-induced NETs occurs in a *P. aeruginosa* elastase-dependent manner. The potential contributions of neutrophil elastase to BPI cleavage require further examination. In addition, CTBPI protein fragments were detected in bronchoalveolar lavage samples of patients with anti-CTBPI autoreactivity (Figure 4D).

Clinical correlations of autoimmunity in CF. The relationship between anti-BPI reactivity and clinical and serologic features in our adult CF cohort was examined (Table 2 and Supplemental Table 3). We observed a significant negative correlation between anti-CTBPI IgG titers and forced expiratory volume in 1 second (FEV1 percent predicted, $r = -0.66$, $P < 0.0001$) (Figure 5A), which demonstrated that anti-CTBPI IgG autoantibodies trend with

Differential BPI cleavage occurs in a P. aeruginosa strain-dependent manner. The C-terminal domain of BPI mediates the interaction of bacterial proteins with DCs (42). Based on our finding that anti-BPI IgG reactivity is specific to the CTBPI, we postulated that BPI cleavage might facilitate its delivery to the DCs in the context of NETosis and so lead to the breaking of tolerance. Moreover, the association of BPI autoantibodies and CF prompted us to examine the potential role of *P. aeruginosa* in BPI cleavage. Following incubation of neutrophils with *P. aeruginosa* strain PA14, immunoblotting with a mouse monoclonal anti-human BPI antibody specific for aa 227–254 revealed the appearance of multiple proteins smaller than 55 kDa, including an appropriately 30-kDa fragment that corresponds to CTBPI (Figure 4A). This effect appeared specific for PA14, as it was not seen with NET induction by PMA or glucose oxidase treatment (Figure 4A). Similar, dose-dependent, effects were seen with different *P. aeruginosa* MOI (PA14 or PAO1) (Figure 4B). Because an elastase cleavage site separates the N-terminus and the

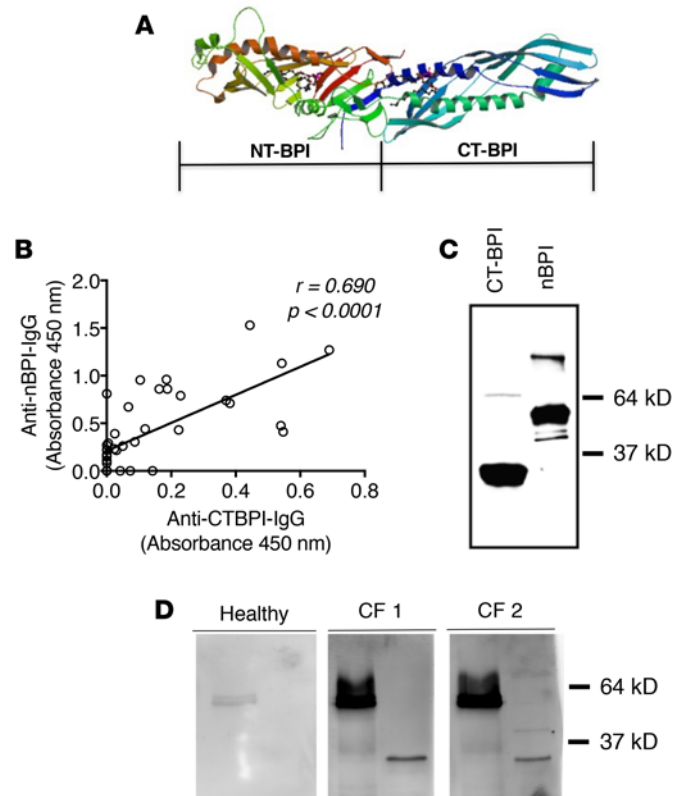


Figure 3. Anti-BPI autoantibodies in CF target the C-terminus of BPI. (A) Crystal structure of BPI protein (RCSB Protein Data Bank, accession number P17213.4); diagram shows N-terminus (NT-BPI, aa 10–240) and C-terminus (CTBPI, aa 260–430). **(B)** Reactivity of cystic fibrosis (CF) sera to the recombinant CTBPI correlates with reactivity to the intact nBPI, as determined by Pearson correlation analysis ($r = 0.690$, $P < 0.0001$, $n = 38$). **(C)** Recombinant CTBPI and nBPI (1 μ g) were detected by immunoblot using monoclonal anti-BPI antibody specific for the epitope mapping to aa 227–254. **(D)** Representative immunoblots confirm reactivity of CF sera to the total BPI (55 kDa, left lane) and to recombinant CTBPI protein (~30 kDa, right lane). nBPI = neutrophil-purified bactericidal permeability-increasing protein.

Table 2. Clinical characteristics of CF patient cohort

Patient population	<i>n</i>	Percentage (%)	FEV1 percent predicted (Mean ± SD)	Age (yr) (Mean ± SD)
Total	38			
Males	25	66%	68 ± 27	32 ± 11
Females	13	34%	59 ± 21	30 ± 6
Age (yr)				
Mean	30.9	-	-	-
Range	18–63	-	-	-
CFTR mutation class				
Class I	5	13.2%	44 ± 27	41 ± 17
Class II	24	63%	62 ± 27	28 ± 8
Class III	2	5.3%	60 ± 5	34 ± 1
Class IV	1	2.6%	26	19
Class V	5	13.2%	50 ± 28	37 ± 6
Unclassified	1	2.6%	23	24
FEV1 percent predicted				
>80%	9	24%	-	26 ± 5
50–80%	12	32%	-	28 ± 5
<50%	17	45%	-	35 ± 13
<i>P. aeruginosa</i> infection				
Positive	29	76%	52 ± 26	32 ± 11
Negative	9	24%	69 ± 26	28 ± 8

Class I: F508del/G542X, F508del/621+1G>T, F508del/711+1G>T, F508del/3659delC, UNKN/1717-1G>T. Class II: F508del/F508 del. Class III: F508del/G551D. Class IV: F508del/H1085R. Class V: F508del/A455E. Unclassified: F508del/EX6b_10dup.

deteriorating lung function in adult CF patients. Similar correlation was found with anti-nBPI autoantibody titers ($P = 0.003$, $r = -0.468$, data not shown) as well as with FEV1 Z score values ($n = 19$, $P = 0.071$, $r = -0.40$, data not shown). Significantly higher autoantibody levels were also detected in patients who experienced more than two pulmonary exacerbations per year ($P = 0.011$, data not shown). Presence of these autoantibodies in the bronchoalveolar lavage fluid of CF patients suggests that the autoimmune response might arise locally in the CF patient airway (Figure 5B). Moreover, incidence of anti-CTBPI IgG strongly associated with *P. aeruginosa*-positive sputum culture as well as with infection with the mucoid form of *P. aeruginosa*, which is associated with increased morbidity and mortality in CF (Figure 5, C and D) (45). Furthermore, patients that were homozygous for the F508 deletion mutation (F508del) in *CFTR* had significantly lower levels of serum anti-nBPI IgG than CF

patients with F508del heterozygosity or a different *CFTR* mutation (Figure 5E). As with the associations of anti-BPI autoimmunity with lower FEV1 percent predicted scores, we observed a trend toward better pulmonary function (i.e., higher FEV1 percent predicted) in patients with F508 deletion homozygosity ($62\% \pm 27\%$) than in patients with other mutation classes ($47\% \pm 24\%$) ($P = 0.09$). (Table 2 and data not shown). Similarly, F508 deletion homozygous patients had a lower prevalence of *P. aeruginosa* infection (70.8%) than patients with a different *CFTR* mutation class (85.7%) (data not shown).

Our model proposes that the breaking of tolerance to BPI is facilitated by *P. aeruginosa*. Interestingly, BPI autoreactivity was associated with increased levels of antibodies toward the CFTR inhibitory factor (Cif) (Figure 5F), a *P. aeruginosa* virulence factor. No relationship was seen with anti-PA14 antibody responses, despite higher levels of anti-PA14 IgG in CF sera (Supplemental Figure 3, A and B). Similarly, we observed no relationship between anti-BPI autoantibody levels and total serum IgG titers, indicating that autoreactivity to BPI is not a consequence of hypergammaglobulinemia or diffuse dysregulation of the humoral immune response (Supplemental Figure 3C). As previously reported, higher levels of total IgG were seen in CF sera relative to the healthy controls (Supplemental Figure 3D) (46). A significant but less striking relationship was also seen between ACarPA and the clinical parameters of disease progression, diminished lung function (FEV1 percent predicted, $r = -0.278$, $P = 0.046$) and *P. aeruginosa* infection ($P = 0.01$) (Supplemental Figure 4, A–C). Intriguingly, the associations of ACarPA in CF patients were detected irrespective of anti-Cif IgG presence (Supplemental Figure 4D).

Discussion

In this article, we report that BPI and carbamylated proteins are associated with NETs, as has been previously observed with citrullinated proteins (47). Second, we demonstrated that autoantibodies to BPI and carbamylated, but not citrullinated, proteins occur in CF patients. However, anti-BPI autoantibodies and ACarPA do not exhibit patterns of coexpression or cross-reactivity in CF patient sera. Third, we showed that antineutrophil BPI autoreactivity maps to the CTBPI and does not appear to require posttranslational modification. This is the first study to our knowledge to show anti-BPI IgG reactiv-

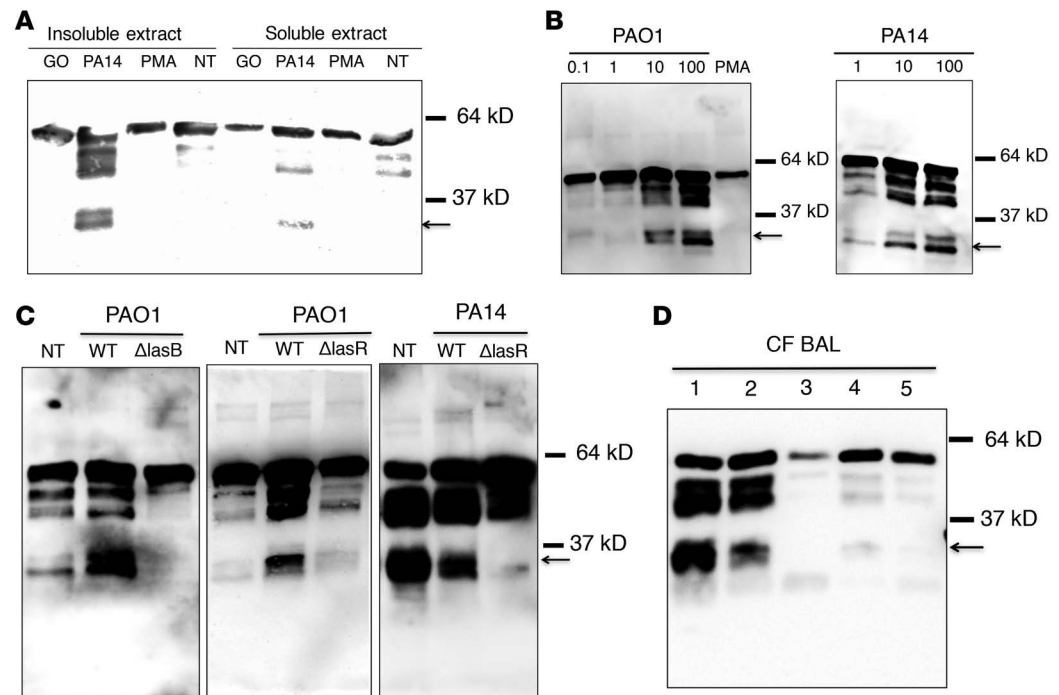


Figure 4. BPI is cleaved in a *P. aeruginosa* strain-dependent manner. (A) Healthy neutrophils were treated with NET-inducing agents, PMA (20 nM), *P. aeruginosa* PA14 (100 MOI), or glucose oxidase (GO, 2 U/ml), or left untreated (NT) for 1 hour. Total BPI protein (55 kDa) and smaller protein fragments were detected in 20 μ g of insoluble and soluble protein extracts via immunoblot with a mouse anti-human BPI antibody directed at aa 227–254 epitope (i.e., BPI C-terminus hinge region). (B) Total BPI and protein fragments were detected by immunoblot in soluble protein extracts (10 μ g) from neutrophils treated with PMA or increasing MOIs of *P. aeruginosa* strains PAO1 (0.1, 1, 10, and 100 MOI) and PA14 (1, 10, and 100 MOI) with the antibody used in A. (C) The extent of BPI cleavage detected in soluble protein extracts (10 μ g) from neutrophils following incubation with *P. aeruginosa* strains PAO1 and PA14, wild type strains (WT), or elastase deficient strains (Δ lasB or Δ lasR) (10 MOI) for 1 hour. (D) Total BPI and protein fragments were detected by immunoblot in BAL samples (20 μ g protein/sample) with the antibody used in A. The immunoblots in A–C are representative images of $n = 3$ experiments. NET, neutrophil extracellular trap; BPI, bactericidal permeability-increasing protein; BAL, bronchoalveolar lavage.

ity to an isolated recombinant BPI C-terminus. Fourth, we found that neutrophil BPI is cleaved in a *P. aeruginosa* elastase-dependent manner, which provides a possible model for the breaking of tolerance to BPI. Finally, in the first-ever analysis of autoimmunity in an adult US CF cohort, we demonstrated that autoreactivity to BPI reflects diminished airway function, which arises in the context of *P. aeruginosa* infection and the *CFTR* genotype.

Since the breaking of tolerance in RA involves lung injury themes common to CF (airway irritants, bronchial inflammation findings on chest CT, and autoantigen-decorated NETs), we initially hypothesized that autoimmunity in CF might parallel the autoantibody profile in RA (48–52). However, anti-BPI IgG and ACPA segregated with CF and RA patients, respectively. In a panel of RA sera enriched for ACPA and ACPA seropositivity, we observed a lower frequency of anti-BPI autoantibodies in RA patients than previously reported in a serologically undefined RA patient population (53). In RA, there is a strong association of ACPA with ACPA (24). In contrast, despite seeing ACPA in CF patients, we observed a poor correlation between ACPA and anti-BPI autoantibodies. Moreover, neither autoantibody associated with hypergammaglobulinemia, suggesting that autoantibody development was not due to a nonspecific increase in B cell growth and differentiation. Additionally, in contrast to RA, we showed that autoimmunity to BPI in CF does not appear to result from posttranslational modifications. Since persistent bacterial infections are one of the major triggers of chronic inflammation in the CF airway, we explored the relationship between *P. aeruginosa* infection, which is present in up to 85% of CF patients, and the development of autoreactivity to BPI.

This is the first study to our knowledge to shed light onto the potential mechanisms involved in the breaking of tolerance to BPI. We demonstrate that *P. aeruginosa* elastase may be the culprit in generating

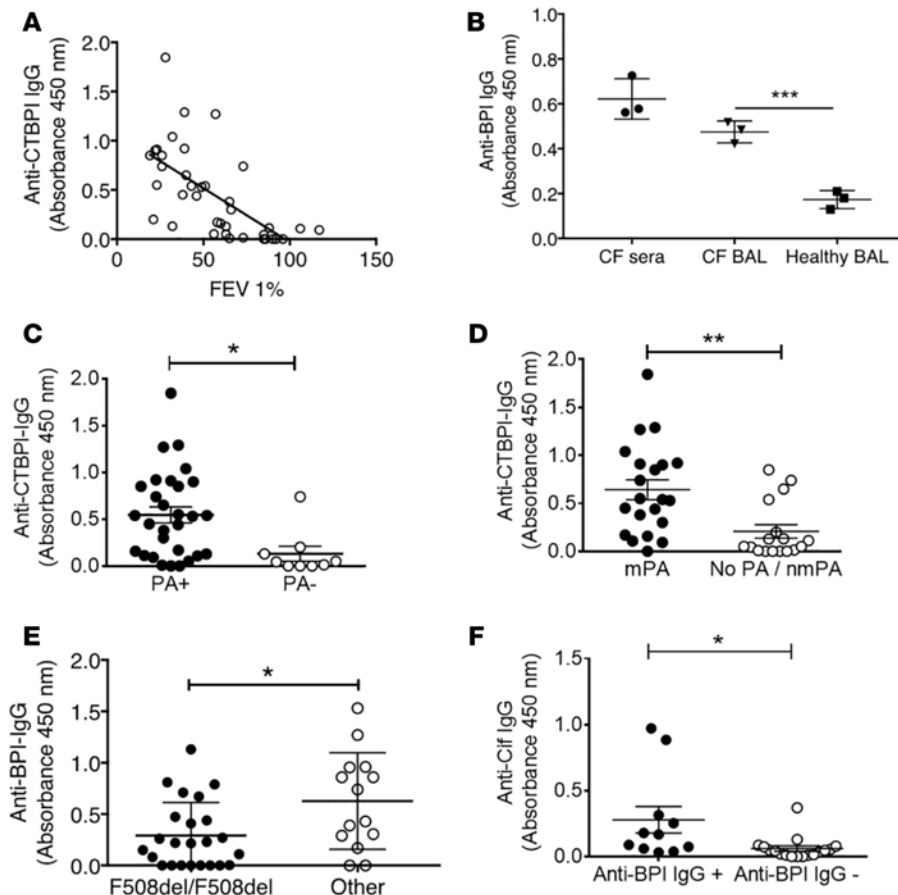


Figure 5. Anti-BPI IgG correlates with FEV1 and anti-Cif immune response to *P. aeruginosa* in a US adult CF cohort. (A) Anti-CTBPI IgG levels determined by ELISA negatively correlate with pulmonary function in cystic fibrosis (CF) patients (FEV1 percent predicted), as determined by Pearson correlation analysis ($n = 38$, $r = -0.664$, $P < 0.0001$). *P. aeruginosa* infection status of patients is denoted: *P. aeruginosa*⁺ (white circle) and *P. aeruginosa*⁻ (black circle). (B) Anti-BPI IgG titers were measured in representative CF bronchoalveolar lavage (BAL) samples that previously demonstrated serum anti-BPI reactivity by ELISA (ANOVA, $n = 3$), $***P < 0.001$. (C) Higher anti-CTBPI IgG levels associate with positive *P. aeruginosa* sputum culture (PA⁺) in CF patients ($n = 38$), (D) particularly with presence of the mucoid *P. aeruginosa* strains (mPA) ($n = 38$). (E) Anti-BPI IgG titers are higher in CF patients that do not carry the F508del homozygous *CFTR* gene mutation ($n = 38$). (F) Anti-Cif (10 $\mu\text{g/ml}$) IgG levels are higher in CF patients with anti-BPI autoantibodies (Anti-BPI IgG⁺) compared with patients without reactivity to BPI (Anti-BPI IgG⁻) ($n = 28$). (C–F) Significance between two groups was determined by Student's *t* test with Welch's correction; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; error bars represent mean \pm SEM. FEV1%, forced expiratory volume in 1 second percent predicted; Cif, CFTR inhibitory factor; BPI, bactericidal permeability-increasing protein.

neopeptides, via BPI cleavage. Based on the studies using the recombinant BPI, the target epitope is likely located in the protein C-terminus, i.e., an appropriately 30-kDa protein fragment detected following neutrophil treatment with wild-type *P. aeruginosa* strains. Our findings support the model in which BPI protein fragments are taken up by the airway antigen-presenting cells (e.g., DCs and monocytes), which leads to the activation of the adaptive immune response and, consequently, to the triggering of autoimmunity (14). In addition to the mechanistic relationship between *P. aeruginosa* elastase and BPI protein integrity, we observed a strong association of BPI autoreactivity with positive *P. aeruginosa* sputum culture, particularly with the more pathogenic mucoid strains. Moreover, presence of autoimmunity to BPI was concurrent with a humoral response directed at Cif (anti-Cif IgG), a virulence protein secreted by *P. aeruginosa* to inhibit CFTR activity (54–56). As expected, these associations were not apparent with ACarPA, which suggests that autoreactivity to BPI occurs specifically in the context of *P. aeruginosa* infection in CF. Adding to the complexity was the observation that high titers of anti-BPI autoantibodies associated with the absence of homozygous F508del *CFTR* mutation. These findings are interesting, as no direct association between the *CFTR* mutation type and the extent of airway disease has been discovered thus far (57). Moreover, little is known about the role different *CFTR* mutation classes play in the intrinsic immune functions and responses in CF patients, particularly in the *P. aeruginosa*-infected lung (58). Our findings suggest that the *CFTR* mutation and particular *P. aeruginosa* strains may interact in the breaking of tolerance to BPI. However, we acknowledge that HLA genotyping might provide an additional insight into the susceptibility of CF patients to develop anti-BPI and/or ACarPA reactivity. In RA, ACPA formation appears highly associated with HLA-DRB1 haplotype (59). In contrast, ACarPA do not appear to share this association in RA (60). It is possible that as-yet undefined HLA haplotypes are associated with anti-BPI and ACarPA responses (61, 62).

The variability in the extent of airway compromise and damage in CF patients remains unexplained. High titers of anti-BPI autoantibodies associated with worse lung function, sputum culture positivity for the mucoid strain of *P. aeruginosa*, and the absence of homozygous F508del *CFTR* mutation. Lack of a

functional BPI has been associated with decreased clearance of the mucoid strain of *P. aeruginosa*, which leads to biofilm formation and obstructed respiratory function (16). Furthermore, anti-BPI autoantibodies have been shown to neutralize the bactericidal and opsonic activity of BPI (63, 64). Therefore, levels of anti-BPI autoantibodies might influence airway compromise by neutralizing the antibacterial role of BPI. Moreover, the presence of chronic infection supports our model of a *P. aeruginosa*-driven mechanism of the breaking of tolerance to BPI. The presence of these autoantibodies in the bronchoalveolar lavage fluid of CF patients is consistent with the hypothesis that local airway infection with elastase-expressing *P. aeruginosa* drives the development of this autoimmunity. *P. aeruginosa* has previously been shown to inactivate host immune responses in the CF lung by proteolytic cleavage of antibacterial peptides and proteins involved in airway homeostasis (65, 66). However, our findings do not exclude a role for neutrophil elastase in BPI cleavage, which is an important contributor to lung damage in CF (67).

In contrast, we note that another autoantibody type, ACarPA, demonstrates a moderate but noteworthy correlation with diminished pulmonary function (lower FEV1) and infection with *P. aeruginosa*. In contrast to anti-BPI responses, the lack of association between ACarPa and anti-Cif IgG levels suggests that ACarPA arise independently of infection and are most likely a marker of neutrophil-driven bronchial inflammation reported in other chronic diseases (24, 68, 69). Alternatively, it may be that ACarPA influence clinical phenotype by impairing the antibacterial activity on NETs. Some limitations of our study are noted. The cohort size and the confounding effect of patient age may have influenced the associations of anti-BPI autoantibodies and ACarPA with clinical parameters. In particular, a larger analysis, including pediatric CF patients, would provide a better understanding of the role CFTR mutation class plays in the development of autoimmunity. Such a study would also begin to address any potential confounding role that age might play in autoimmunity. In addition, examining the full repertoire of autoantibodies in CF patients would provide a more complete picture of the immune response, as anti-PR3 autoantibodies have been reported in pediatric CF patients (70).

Our study provides insight into the development of autoimmunity to BPI, as well as to carbamylated proteins, in CF. Even though recurring flares lead to irreversible lung damage, no correlation has been found between changes in the microbiome and disease exacerbation (71). This study suggests that the level of anti-BPI autoantibodies can serve as a marker of deteriorating lung function in a subset of CF patients. Further studies will be required to understand the possible contributions of these autoantibodies to disease progression. Moreover, this work reveals a likely mechanism that leads to the breaking of tolerance in an environment of chronic *P. aeruginosa* infection, which has previously been identified as one of the triggers of autoimmune disease (72, 73). Future epitope mapping will determine the immunogenic peptide of the BPI protein. These studies could also provide an insight into the development of autoimmunity to BPI in other inflammatory lung diseases, such as bronchiectasis and interstitial lung disease (74, 75). Due to the complexity of the inflammatory environment in the lung, future studies of anti-BPI autoimmunity development and function should also address the role of neutrophil elastase, particularly its ability to cleave and degrade immunoglobulins (76, 77).

Methods

Patient cohorts. Sera were collected from a cohort of 38 adult CF patients treated at Dartmouth-Hitchcock Medical Center (DHMC), which is located in Lebanon, New Hampshire, USA. The mean age of the CF patients was 30.9 years (range 18–63 years), and 25 patients were male, while 13 were female. Sputum cultures and patient lung function at the time of serum collection were assayed by clinical laboratories at DHMC. No patient has undergone lung or liver transplantation. RA patient sera (94% seropositive for ACPA and IgM rheumatoid factor; $n = 50$) were stored in the Rheumatic Disease Biomarker Biorepository at DHMC.

Antibody detection. The frequency of seropositivity of the RA and CF patient cohorts was measured by CCP3 and the QUANTA Lite RF IgM ELISA kit (Inova Diagnostics), while serum IgG levels were measured using the Human IgG ELISA kit (ICL Inc.). Cif (a gift of Dean Madden at Geisel School of Medicine at Dartmouth) and *P. aeruginosa* PA14 lysates were used to create ELISA assays for measurement of antibodies to these proteins in CF and normal human sera (1:100 dilution).

Anti-BPI autoreactivity was measured in patients with CF and RA, and in healthy subjects, by an in-house ELISA coated with nBPI (Athens Research and Technology), which was validated ($R^2 = 0.85$) against the commercial anti-BPI ELISA assay (Immuno-Biological Laboratories Inc.). After being coated with human nBPI (10 $\mu\text{g}/\text{ml}$), 96-well ELISA plates were blocked with PBS+1% BSA overnight. Sera,

diluted 1:100 in the dilution buffer (PBS+1%BSA), or undiluted bronchoalveolar lavage fluid were incubated for 1 hour at room temperature. Anti-human IgG (H+L), F(ab')₂ fragment, and peroxidase-labeled antibody (KPL Inc.) (1:50,000) were added, followed by R&D Systems substrate and stop solution. Absorbance was read at 450 nm using ELISA reader (Epoch, BioTek). The presence of anti-BPI IgG in CF sera was verified by fluid-phase inhibition ELISA, where 50 µg nBPI was used as inhibitory agent (30 minutes at room temperature), prior to the ELISA assay. A minimum of a 30% reduction in OD_{450nm}, relative to the serum control, was considered meaningful (Supplemental Figure 5). Autoantibodies to CTBPI were also measured by ELISA. The recombinant CTBPI was produced by TOPO Cloning technology (ThermoFisher Scientific). The primers used for the pET1D1/D-TOPO-6XHis tag vector are as follows: 5'CATGAACAGCCAGGTCTGCGAGA3' (upper) and 5'TTTATAGACAACGTC TGCACCG3' (lower). Nickel-coated ELISA plates (ThermoFisher Scientific) were used to bind the 6X-His tagged CTBPI (10 µg/ml), and ELISA was performed as described for nBPI. Anti-carbamylated protein IgG titers (ACarPA) in sera were evaluated by commercial ACarPA ELISA assay (Inova Diagnostics Inc.) as well by a previously described in-house ACarPA ELISA (24). Positive cutoff for the BPI ELISA assays was defined as mean + 2 SDs of the healthy donor cohort.

Detection of autoantibody reactivity by immunoblot. Immunoblotting of nBPI, native fibrinogen (Sigma-Aldrich), and carbamylated fibrinogen (1 µg) was performed after resolution by SDS-PAGE (12% acrylamide gel) and transfer to a nitrocellulose membrane (Sigma-Aldrich). Following blocking in tris buffer saline with 0.05% tween-20 (TBS-T) + 3% BSA, the membranes were probed with patient serum (1:100 in TBS-T + 1% BSA) for 1 hour at room temperature, washed, and incubated with goat anti-human peroxidase-labeled secondary antibody (1:400,000, in TBS-T + 1% BSA). Enhanced chemiluminescent substrate SuperSignal West Femto (ThermoFisher Scientific) was used for protein detection via the Syngene G-Box system and software (Synoptics). A slightly modified protocol was utilized to subsequently detect reactivity of CF sera to nBPI and CTBPI via immunoblot: nitrocellulose membranes were blocked in TBS-T + 10% FCS overnight and probed with CF sera (1:250, in TBS-T + 3% FCS) for 2 hours; goat anti-human peroxidase-labeled secondary antibody was used at 1:6,000 dilution; and the enhanced chemiluminescent substrate SuperSignal West Pico (ThermoFisher Scientific) was used for detection.

NET induction and immunocytochemistry. Induction and visualization of NETs was done as previously described (78). Following Ficoll discontinuous gradient separation, neutrophils were purified from the erythrocyte pellet by 5% dextran sedimentation. Neutrophils were seeded onto glass coverslips (2 × 10⁵ cells), activated with 600 nM PMA for 2 hours, and fixed in 4% paraformaldehyde. Rabbit anti-human neutrophil elastase antibody (Abcam) and rabbit anti-BPI antibody (Sigma-Aldrich) were directly labeled with Alexa Fluor 488 and Alexa Fluor 568 protein labeling kits (ThermoFisher Scientific), respectively; 100 µg/ml anti-neutrophil elastase-AI488 and 50 µg/ml anti-BPI-AI568 were used for immunocytochemistry. BPI was also detected using mouse anti-BPI antibody (1:200, Santa Cruz Biotechnology Inc.), followed by donkey anti-mouse Alexa Fluor 488 antibody (1:500, Jackson ImmunoResearch). Carbamylated and citrullinated proteins were detected using rabbit anti-carbamyl lysine antibody (αCBL, Cell Biolabs Inc.) and rabbit anti-citrulline antibody (Abcam) (1:200), followed by donkey anti-rabbit Cy3 antibody (1:500, Jackson ImmunoResearch). Samples were mounted using ProLong Gold Antifade Mount with DAPI (ThermoFisher Scientific) and visualized with the laser point scanning confocal microscope (LSM 510 META, Zeiss; ×60). Donkey anti-rabbit Cy3 and donkey anti-mouse AI488 secondary antibodies were used alone to determine non-specific staining (Figure 1, B and C).

Detection of BPI posttranslational modifications. A 55-kDa band corresponding to purified nBPI (10 µg, Athens Research and Technology) was resolved by a 12% SDS-PAGE gel; the band was cut out and sent for proteomic analysis for citrullinated and carbamylated posttranslational modifications by tandem mass spectrometry at the Mass Spectrometry & Proteomics Resource at the W.M. Keck Foundation Biotechnology Resource Laboratory (Yale School of Medicine). Isoaspartylation (isoAsp) in purified BPI protein was measured by isoAsp vapor assay as described previously (79). 50 pmole isoAsp containing δ sleep-inducing peptide (isoAsp-DSIP) was used as a positive control, and a reaction mix without isoAsp-DSIP was applied as a negative control. The presence of posttranslational modifications of BPI was also investigated using immunocytochemistry, as described above.

Detection of BPI cleavage by P. aeruginosa. P. aeruginosa PA14 and PAO1 strains were provided by Brent Berwin at the Geisel School of Medicine at Dartmouth. The mechanism of BPI cleavage was investigated using PAO1 *AlasR* and PA14 *AlasR* bacterial strains provided by Deborah Hogan at the Geisel School of

Medicine at Dartmouth as well as using the PAO1 *AlsB* strain, a gift of Dao Nguyen laboratory at McGill University, Montréal, Quebec, Canada (44, 80). PMNs from healthy human donors (1×10^6 cells/ml) were treated with PMA (20 nM), glucose oxidase (2 U/ml), or *P. aeruginosa* PA14 (10 MOI) for 1 hour at 37°C. Soluble and insoluble protein extracts were prepared as previously described (81), protein concentration was measured by the Pierce BCA Protein Assay Kit (ThermoFisher Scientific), and BPI was detected by immunoblot in 20 µg of protein extract, using a mouse monoclonal anti-human BPI antibody specific for the epitope mapping between aa 227 and aa 254, i.e., the elastase cleavage site of BPI C-terminus (1:1,000) and goat anti-mouse peroxidase-labeled secondary antibody (1:100,000).

Statistics. Data were analyzed using GraphPad Prism 6 software (GraphPad Software Inc.). Two-tailed Student's *t* test, with Welch's correction, was applied to determine the significance in the difference between two data sets. One-way ANOVA, followed by Bonferroni post-hoc, was used to compare means between multiple data sets. Pearson coefficient correlation analysis was used to determine the strength of the relationship between two parameters. *P* values of less than 0.05 were considered significant.

Study approval. The Dartmouth College Committee for the Protection of Human Subjects (CPHS) approved the use of patient material for this study. Written informed consent was obtained from patients, and patient samples were deidentified.

Author contributions

SS and WFCR designed the research studies. AA and AHG provided the patient samples and critically reviewed the manuscript. SS and BJH conducted the experiments. MLY and MM performed proteomic analyses. SS and BJH acquired and analyzed the data. JDJ assisted with data analysis and critically reviewed the manuscript. SS and WFCR wrote the manuscript.

Acknowledgments

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award P30GM106394. We thank Brent Berwin, Iviana Torres, Sally Demirdjian, Deborah Hogan, John Hammond, Dao Nguyen, and Geoffrey McKay for providing bacterial strains and for their microbiology expertise. We thank the Translational Research Core at the Dartmouth Geisel School of Medicine, and Amanda Nymon for procuring and handling the CF samples. Microscopy was carried out at the Norris Cotton Cancer Center Microscopy Shared Resources facility with the help of Radu Stan and Kenneth Orndorff. We also thank Yina Huang for her invaluable contributions to the preparation of this manuscript.

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