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Systemic levels of anti-PAD4 autoantibodies correlate with airway obstruction in cystic fibrosis

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Abstract

Cystic fibrosis (CF) airway disease is characterized by the long-term presence of neutrophil granulocytes. Formation of neutrophil extracellular traps (NETs) and/or autoantibodies directed against extracellular components of NETs are possible contributors to neutrophil-mediated lung damage in CF. The goal of this study was to measure their levels in CF adults compared to healthy controls and subjects with rheumatologic diseases known to develop NET-related autoantibodies and pathologies, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Sera were analyzed from the following number of subjects: 37 CF, 23 healthy controls (HC), 20 RA, and 21 SLE. CF had elevated serum myeloperoxidase (MPO) concentrations (347.5+/−56.1 ng/ml, mean +/-S.E.M., p=0.0132) compared to HC (144.5+/−14.6 ng/ml) but not of neutrophil elastase (NE) complexed with alpha-1-antitrypsin, cell-free DNA or NE-DNA complexes. The peptidylarginine deiminase 4 (PAD4) enzyme is required for NET formation and associated DNA release in neutrophils. Serum levels of anti-PAD4 antibodies (Ab) were elevated in CF (p=0.0147) compared to HC and showed an inverse correlation with a measure of lung function, FEV1% predicted (r = −0.5020, p=0.015), as did MPO levels (r = −0.4801, p=0.0026). Anti-PAD4 Ab levels in CF sera associated with lung infection by P. aeruginosa, but not that by S. aureus, age, sex, CF-related diabetes or the presence of musculoskeletal pain. Serum levels of anti-citrullinated protein Abs

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Conflict of interest statement
The authors have no conflict of interest related to this work.
(ACPAs) and anti-nucleosome Abs were not elevated in CF compared to HC (p=0.7498, p=0.0678). In summary, adult CF subjects develop an autoimmune response against NET components that correlates with worsening lung disease.

Keywords
Cystic fibrosis; neutrophils; neutrophil extracellular traps; PAD4; autoantibody; lung disease; autoimmunity

Introduction
Neutrophil recruitment to and chronic polymicrobial bacterial infections of the airways are major pathologic drivers of progressive lung disease in cystic fibrosis (CF) and thus contribute to mortality and morbidity (1). CF airway neutrophils cause damage in two ways - by failing to clear pathogenic bacteria and by releasing toxic material into the extracellular environment. For example, the amounts of neutrophil elastase (NE) detected in supernatants of CF airway fluid samples or on the surface of CF sputum neutrophils correlate with the degree of airway obstruction (2, 3). CF airway levels of myeloperoxidase (MPO), another primary granule marker of neutrophils, also correlate with lung dysfunction (4). Extracellular DNA derived from host neutrophils is abundant in CF airways, and also correlates with airflow obstruction (5–8). Thus, neutrophils are believed to mediate CF lung tissue damage by release of their intracellular content into the airways.

More recently, attention has been focused on another mechanism whereby activated neutrophils can cause airway damage in CF which is formation of neutrophil extracellular traps (NETs) (9). NETs are large extracellular complexes extruded by the neutrophil and are composed of a DNA scaffold associated with histones and granule components, key of which are NE and MPO (9, 10). P. aeruginosa, a major respiratory pathogen in CF, induces robust NET formation (10, 11). While NETs are present in large quantities and most neutrophils undergo NET release in CF airways (12, 13), the exact role of NETs in progression of CF lung disease remains unclear. Measuring extracellular DNA levels in CF airway fluids is insufficient to prove a role of NETs in CF lung disease as DNA may originate from neutrophils dying by mechanisms other than NET formation or from cells other than neutrophils.

Citrullination of histones by peptidylarginine deiminase 4 (PAD4) is a hallmark of NET release, as it does not occur in apoptotic or necrotic cells (14, 15). During NET formation, arginine residues in histones are post-translationally converted into citrulline by PAD4 (11, 15). Human PMNs express large amounts of PAD4 (16). Citrullinated histones are present in NETs but not in resting PMNs (17) and are also found in CF airways (12, 13). PAD4 mediates NET release via histone hypercitrullination inducing heterochromatin decondensation, chromatin unfolding and swelling (14, 18–20). In addition to their potential direct contribution to lung damage, NETs may also serve as chronic sources of autoantigens in CF (21). The chronic presence of NETs and neutrophil components in CF airways could trigger an autoimmune response in the host and immune complexes could be the drivers of airway damage. Although the damage is occurring in the airway, we wanted to explore a...
more accessible space, the blood, for markers of both NET formation and autoantibodies against NETs, and how this may correlate with the degree of lung disease. Therefore, the goal of this study was to measure the degree of NET formation, as well as, levels of autoantibodies in CF young adults compared to healthy controls and subjects with rheumatologic diseases known to develop NET-related autoantibodies and pathologies, rheumatoid arthritis (RA) and systemic lupus (SLE). We hypothesized that CF subjects would show evidence of NET formation in the blood, as well as, an autoimmune response to the extracellular components of NETs. Furthermore, we hypothesized that the degree of NETosis and of an autoimmune response would correlate with the degree of airway dysfunction in CF.

Materials and Methods

Control human subjects

The human subject studies were performed by following the guidelines of the World Medical Association’s Declaration of Helsinki. Control human subjects were recruited at the University of Georgia to donate blood for serum preparation. The human blood protocol (UGA# 2012–10769-06) and the consent form were reviewed and approved by the Institutional Review Board (IRB) of the University of Georgia. Enrolled healthy volunteers provided informed consent prior to blood draw. The age and sex of the healthy donors were matched to those of CF patients (Table 1). Ten milliliters of blood were drawn into a silicone coated tube and allowed to clot at room temperature for 30 minutes. The tube was centrifuged, serum aliquotted and stored at −80°C for later analysis.

CF patients

CF subjects were patients followed at the Adult CF Clinic at Emory University who had signed informed consent to provide blood samples and clinical data for the CF Biospecimen Repository in accordance with the Emory University IRB (Emory #00042577). All patients had the diagnosis of CF confirmed by pilocarpine iontophoresis sweat testing and/or CFTR gene mutation analysis showing the presence of two disease causing mutations. Blood was drawn when the subject had been clinically stable for a minimum of three weeks and no new medications had been started during the prior three weeks. Four milliliters of blood were drawn into a silicone coated tube and allowed to clot at room temperature for 30 minutes. The tube was centrifuged, serum aliquotted, and stored at −80°C until shipped to UGA on dry ice for analysis. Sputum cultures in those patients who could expectorate sputum and throat cultures were taken on the day of the blood draw and the presence or absence of Pseudomonas aeruginosa or Staphylococcus aureus as identified by the clinical microbiology laboratory was noted. Baseline lung function was defined as is done for the CF Foundation Patient Registry which is the average of the best percent predicted forced expired volume in one second (FEV₁) for each quarter of the calendar year. Blood and clinical data were collected during the period of 2010 to 2016.

SLE patients

Systemic lupus erythematosus (SLE) patients meeting four or more American College of Rheumatology lupus criteria (22) were recruited at the University of Michigan lupus clinic.

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under IRB-Med 00066116. All patients underwent written, informed consent and were treated according to the declaration of Helsinki. Five mL of blood was collected from SLE patients in a serum separator tube, aliquoted and frozen at −80°C. They were sent on dry-ice to UGA for analysis.

**Patients with Rheumatoid Arthritis**

Serum from rheumatoid arthritis (RA) patients attending rheumatology clinics were obtained from the University of Alabama at Birmingham (UAB) Rheumatology Arthritis Database and Repository (RADAR). All patients met the 1987 ARA (now ACR) or 2010 ACR/EULAR classification criteria. All data and samples were obtained in accordance with the UAB IRB. Standard techniques for venipuncture and isolation of serum were used. Serum was processed from SST tubes almost exclusively on the same day as venipuncture. Serum samples were aliquoted, frozen at −80°C and shipped on dry ice to UGA to minimize freeze/thaw cycles.

**Myeloperoxidase and neutrophil elastase ELISA**

Commercial ELISA kits were used to determine serum levels of MPO (R&D Systems, DuoSet, Minneapolis, MN, USA) and complexes of NE and alpha-proteinase inhibitor (PI) (Abcam, Cambridge, MA) according to the manufacturers’ instructions. Results are expressed as ng/ml in undiluted human serum. The experimental limits of detection were: 125.0 pg/ml (MPO ELISA) and 78.1 pg/ml (NE/alpha1-PI ELISA).

**DNA quantification**

The concentration of cell-free double stranded DNA in human serum samples was quantitated with the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Grand Island, NY, USA). Serum samples were diluted 10 to 100–fold in sterile PBS and PicoGreen reagent was added according to manufacturer’s instructions. DNA doses were quantitated using a known DNA standard and are expressed as ng/ml in undiluted human serum.

**Quantification of NETs**

NETs in human serum samples were quantitated as complexes of human neutrophil elastase and DNA using a non-commercial ELISA kit established at UGA previously (11, 23). Briefly, 96-well ELISA plates were coated with a capture antibody against human neutrophil elastase, blocked, exposed to diluted serum samples and treated with a detection antibody against double stranded DNA conjugated with horseradish peroxidase. Plates were washed three times with PBSTween20 solution between each of these steps. Signal was developed using TMB substrate and measured at 450 nm using microplate photometer (Eon, Biotek Instruments, Winooski, VT, USA). NET results are expressed as percentage of a semi-quantitative NET-standard composed of NETs of PMA-stimulated human neutrophils pooled from at least five different healthy human donors.
ACPA ELISA

The concentration of IgG class autoantibodies against citrullinated proteins (ACPA) in human serum was determined using an indirect solid phase enzyme immunoassay following the manufacturer’s instructions (Abnova, Taipei City, Taiwan). Serum samples were diluted 50 to 100-fold. Results are expressed as U/ml and the limit of detection is 1 U/ml.

Nucleosome antibody ELISA

Human serum levels of IgG autoantibodies to nucleosomes were quantitated with a commercial ELISA kit following the manufacturer’s instructions (Abnova, Taipei City, Taiwan). Human nucleosomes bound to the ELISA plate bind nucleosome autoantibodies potentially present in the serum samples and horseradish peroxidase-labelled anti-human IgG antibodies were used to develop a colorimetric signal using TMB substrate. The reaction was stopped and absorption was measured at 450 nm. Results are expressed as U/ml in undiluted human serum and the limit of detection of this assay is 20 U/ml.

PAD4 autoantibody ELISA

Serum levels of autoantibodies to PAD4 (anti-PAD4) were measured using the PAD4 Autoantibody ELISA kit following manufacturer’s instructions (Cayman Chemical, Ann Arbor, Michigan, USA). Human serum samples diluted 250 to 5000–fold were added to 96-well ELISA plate precoated with human PAD4. The presence of anti-PAD4 antibodies of any isotype (IgG, IgA, IgM) was detected by adding goat anti-human(H+L) antibodies conjugated with horseradish peroxidase. Colorimetric signal was developed using TMB substrate. An affinity-purified anti-PAD4 antibody isolated from the blood of a rheumatoid arthritis patient was used as a standard in the kit. Results are expressed as U/ml in undiluted human serum and the limit of detection is 31 U/ml.

Statistical analysis

Results between two patient cohorts were analyzed by Mann-Whitney test while data among more than two cohorts were compared by Kruskal-Wallis and Dunn’s multiple comparisons tests. Correlation between two parameters was evaluated with Spearman’s rank-order correlation. Data are expressed as mean plus-minus standard deviation (SD). The correlation coefficient (r) and two-tailed p values were calculated. Statistically significant differences were considered as *, p<0.05; **, p<0.01; ***, p<0.001. Statistical analysis was carried out with GraphPad Prism version 6.07 for Windows software.

Results

Demographics of all subjects studied and clinical characteristics of the CF subjects

Table 1 shows the age and sex distribution of the four cohorts which included 37 CF subjects, 23 healthy controls, 20 subjects with RA, and 21 with SLE. As expected, females dominated the SLE group. Table 2 shows the clinical characteristics of the CF subjects. This cohort is generally representative of a young adult CF cohort with 62% being positive for P. aeruginosa, 75% having at least 1 F508 del allele, and 43% having CF-RD. What is not quite typical is that these young adults tended to have milder airflow obstruction than seen on
average in the US. Indeed, the Adult CF program at Emory consistently ranked in the highest quartile for lung function in adults during the study period. For example, the 2016 CF Foundation Registry shows that the median percent predicted FEV$_1$ for CF adults (i.e. 18 years of age and older) for all CF Centers in the US was 68% whereas the median for Emory was 73%.

**Levels of neutrophil primary granule markers in the CF blood correlate with airflow obstruction**

Neutrophil primary granule components, MPO and NE, are anchored to extracellular DNA in expelled NETs (9) and can be found in CF airways. To assess whether these granule markers can be also detected in sera of CF patients, we performed ELISA assays. CF patients had significantly elevated serum MPO concentrations (347.5+/−56.1 ng/ml, mean+/−S.E.M., n=37, p=0.0132) compared to healthy controls (144.5+/−14.6 ng/ml, n=23) (Fig. 1A). Elevated serum MPO levels were also observed in RA (1,256.8+/−165.4 ng/ml, n=21) and SLE patients (854.7+/−104.2 ng/ml, n=20). Serum NE levels in the blood of CF patients (294.6+/−67.0 ng/ml, mean+/−S.E.M., n=37) were not significantly higher compared to control subjects (92.9+/−21.5 ng/ml, mean+/−S.E.M., n=23) (p=0.0627) (Fig. 1B). Serum MPO levels showed a significant negative correlation (r = -0.4801, p=0.0026) with baseline lung function (Fig. 1C). Serum MPO and NE levels revealed a strong significant correlation with each other (r = 0.9637, p<0.0001) (Fig. 1D). Serum NE concentrations, however, showed a negative, significant correlation with FEV$_1$% (r=-0.5128, p=0.0012). These results confirm that systemic levels of neutrophil azurophilic granule components are indicative of lung disease severity in CF.

**NET and DNA levels in CF serum are not elevated**

Extracellular DNA provides the structural basis of NETs (9). Extracellular DNA and NETs are abundant in CF airways (12, 13). To reveal whether extracellular DNA and NETs can also be detected in the circulation, we quantitated the levels of cell-free DNA in sera of CF patients and control subjects. No significant difference (p=0.7744) was found between mean serum DNA levels of CF patients (1624.6+/−444.3 ng/ml, mean+/−S.E.M, n=37) and control subjects (1286.1+/−111.4 ng/ml, mean+/−S.E.M., n=23) (Fig. 1E). To quantitate serum NETs, we performed an ELISA assay established in our laboratory capable of detecting NET-specific complexes of NE and DNA (11, 23). As results of Figure 1F show, there was no significant difference (p=0.2247) observed between CF (1.31+/−0.42, % of NET-standard, mean+/−S.E.M, n=37) and control sera (1.46+/−0.37, % of NET-standard, mean+/−S.E.M, n=23). Thus, circulating levels of cell-free DNA or NETs are not elevated in CF.

**ACPA levels in CF blood are not elevated**

NETs have been detected in CF airways and one study reported the presence of citrullinated histones by immunofluorescence (12). Citrullination of histones by PAD4 has been implicated in the process of NET formation (14, 15). Citrullinated proteins serve as autoantigens in RA leading to the production of anti-citrullinated protein antibodies (ACPAs) that are a hallmark of the disease (24). ACPAs represent the link between the innate and adaptive immune system in RA (25, 26). NETs and PMNs have been implicated in the pathogenesis of RA (27). The presence of ACPAs indicates that citrullination, as a
posttranslational protein modification performed by PAD enzymes, creates neoantigens that trigger an autoimmune response. Detecting ACPAs in CF would suggest delivery of citrullinated self-antigens to the extracellular environment by PAD4-mediated NET formation to activate autoreactive B cells for autoantibody production. To explore this, we assessed the levels of ACPAs in sera of CF, RA and SLE patients and HC. ACPA levels were not significantly elevated (p=0.0678) in CF sera (4.37+/−0.82 kU/ml, n=37) compared to controls (3.33+/−0.67 kU/ml, n=23) (Fig. 2A). While the overall difference in serum ACPA levels between RA and CF/HC cohorts turned out to be non-significant due to six RA patients with unusually low ACPA levels, sera of RA patients had ACPA levels (13.31+/−4.24 kU/ml, n=21) more than three-fold higher than CF patients (Fig. 2A). Interestingly, SLE patients had significantly lower serum ACPA levels than any of the other three patient cohorts in our study. Thus, a significant general antibody response to citrullinated proteins is not present in CF patients.

**Nucleosome autoantibodies are not elevated in CF blood**

Nucleosomes are also released from neutrophils during NET formation (9). Chronic presence of extracellular DNA and –most likely- nucleosomes in CF airways could also potentially trigger an autoimmune response leading to the production of anti-nucleosome autoantibodies. We measured anti-nucleosome autoantibody levels in sera of CF, RA and SLE patients and healthy controls. SLE is characterized by high levels of chromatin autoantibodies (28) and is used here as a non-CF disease control. We did not observe any significant difference (p=0.7860) in chromatin autoantibody levels between CF samples (2.01+/−0.24 kU/ml, mean+/−S.E.M, n=37) and healthy controls (1.75+/−0.16 kU/ml, mean+/−S.E.M., n=23) (Fig. 2B). As expected, SLE patients had anti-nucleosome autoantibody levels several-fold and significantly higher (27.69+/−11.48 kU/ml, n=21, mean+/−S.E.M.) than CF patients or HC (Fig. 2B). Overall, CF patients do not have elevated levels of autoantibodies against nucleosomes.

**Autoantibodies directed against PAD4 are elevated in CF serum and correlate with lung disease**

PAD4 is an enzyme highly expressed in neutrophils and central to the process of NET formation (14). To reveal whether PAD4 would trigger an autoimmune response in CF, we assessed serum anti-PAD4 antibody levels in CF patients and healthy controls. As presented in Figure 3A, anti-PAD4 antibody levels were significantly higher (p=0.0058) in CF blood (139.47+/−21.37 kU/ml, n=37, mean+/−S.E.M.) compared to non-CF, control serum (54.35+/−4.81 kU/ml, n=23, mean+/−S.E.M.). As expected, anti-PAD4 autoantibody levels were significantly higher in RA sera (267.4+/−65.2 kU/ml, mean+/−S.E.M., p=0.0005) than in control subjects (Fig. 3A). Anti-PAD4 autoantibody levels were not significantly different between CF and RA patients (p>0.999) (Fig. 3A). Anti-PAD4 autoantibody levels in SLE sera were not significantly different from results obtained in any other patient cohort tested (p=0.132 vs HC; p=0.729 vs RA; p>0.999 vs CF) (Fig. 3A). Our most striking results show that serum anti-PAD4 antibody levels in CF patients negatively correlate with lung function (Fig. 3B). CF patients with serum anti-PAD4 antibody levels higher than the maximum value in the control group (> 112.44 kU/ml) have significantly (p=0.0026) worse lung function (FEV₁% pred: 66.2%+/−18.9%, mean+/−S.D., n=16) than CF patients with anti-
PAD4 antibody levels overlapping with control values (FEV$_1$%$_{\text{pred}}$: 85.5%+/−18.8%, mean+/−S.D., n=21). Thus, our results detect elevated levels of anti-PAD4 antibodies in the circulation of CF patients that correlate with worse airflow obstruction.

**Anti-PAD4 antibody serum levels in CF are linked to P. aeruginosa lung infection**

CF airway disease is characterized by chronic, polymicrobial infections. The two most prevalent bacterial pathogens that infect the lungs of most CF patients are *Pseudomonas aeruginosa* and *Staphylococcus aureus*, with co-infections by *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA) being associated with more frequent pulmonary exacerbations and greater decline in lung function (29). To explore potential associations with bacterial lung infections, we grouped our CF cohort according to the presence of either *P. aeruginosa* or *S. aureus* in sputum cultures taken on the day of blood draw, and compared their serum anti-PAD4 antibody levels. We found that CF patients with *P. aeruginosa* had significantly higher serum levels of anti-PAD4 antibodies (176.10+/−31.63 kU/ml, mean+/−S.E.M., n=23) than CF patients without *P. aeruginosa* (79.28+/−14.30 kU/ml, mean+/−S.E.M., n=14, p=0.039) (Fig. 4A) or healthy control subjects (69.66+/−12.03 kU/ml, mean+/−S.E.M., n=23, p<0.001). Interestingly, the difference in anti-PAD4 autoantibody levels between *P. aeruginosa*-negative CF patients and control subjects was non-significant (p=0.662, Fig. 4A).

No significant difference was observed in ACPA or anti-nucleosome autoantibody serum levels when comparing control subjects, *P. aeruginosa*-positive or *P. aeruginosa*-negative CF patients in any combination (data not shown). Although it was statistically not significant (p=0.0547, Mann-Whitney test), there was a trend of decreased lung function in CF patients with *P. aeruginosa* infection compared to those without *P. aeruginosa* in our CF cohort (FEV$_1$, 71.4+/−4.5% in *P. aeruginosa*-infected CF patients, n=23 vs. 86.5+/−4.0% in CF patients without *P. aeruginosa*, n=14, mean+/−S.E.M.) (Fig. 4B). No correlation (p=0.4016) was found, however, between anti-PAD4 antibody levels and the mucoid status of *P. aeruginosa* (Fig. 4C). When CF patients were grouped according to the presence or absence of *S. aureus* lung infection, no significant difference (p=0.3427) was found in serum anti-PAD4 antibody levels: 144.71+/−24.23 kU/ml in *S. aureus*-negative group (n=18) and 134.50+/−36.05 kU/ml in *S. aureus*-positive group (n=19, mean+/−S.E.M.) (Fig. 4D). No significant difference was observed in anti-PAD4 antibody levels between CF patients infected with MRSA and those infected with methicillin-sensitive *S. aureus* (MSSA, Fig. 4E) (p=0.7345). Since co-infections by *P. aeruginosa* and MRSA have been associated with the most severe lung disease in CF patients (30), we compared serum anti-PAD4 antibody levels among *P. aeruginosa*-infected CF patients without *S. aureus*, infected with MRSA or MSSA. No significant differences were found (Fig. 4F).

CF is a heterogeneous disease with complex genetics and several comorbidities (31). The ΔF508 deletion mutation is responsible for about two thirds of the cases with CF (32). Among the 37 CF patients in our cohort, 16 were homozygous for ΔF508, 12 patients had one ΔF508 allele while 9 patients did not have any copy of ΔF508 and carried other CFTR mutations. When CF patients were grouped according to the copy number of the ΔF508 allele, no link (p=0.3927) to serum anti-PAD4 antibody levels was observed (data not shown). Analyzing the data according to CF patients’ sex, no significant (p=0.6171, data not shown) difference was recorded in their anti-PAD4 antibody concentrations: male CF...
patients (128.74+/−23.56 kU/ml, n=21) vs female patients (153.55+/−40.12 kU/ml, n=16, mean+/−S.E.M.). To reveal whether anti-PAD4 antibody levels are associated with joint disease, we compared antibody blood levels in those seven patients who reported joint pain in our CF cohort. We did not find any significant difference in blood anti-PAD4 antibody levels between CF patients with or without musculoskeletal pain (p=0.4803, data not shown). No correlation between anti-PAD4 autoantibody levels and age of CF patients was observed either (r=0.2398, p=0.1528, not significant), although it should be noted that this study involved adults and not children. Finally, since autoimmunity plays a significant role in diabetes, we compared anti-PAD4 autoantibody levels between the CF subjects with CF-related diabetes (CFRD) and those without diabetes. In our cohort, 16 CF patients were diagnosed with CFRD while 20 patients did not have CFRD (there was no related information about one CF patient) (Table 2). No significant difference (p=0.2901) was observed when circulating anti-PAD4 autoantibody levels were compared in CF patients according to the presence or absence of CFRD: 185.89+/−44.18 kU/ml (CFRD-positive) versus 107.65+/−15.46 kU/ml (CFRD-negative, mean+/−S.E.M.) (data not shown). Thus, systemic levels of anti-PAD4 antibodies are not associated with sex, ΔF508 allele copy number, age, CFRD, or joint pain in CF patients. Among the two major respiratory bacterial pathogens in CF, only P. aeruginosa, and not S. aureus, was found to be associated with higher systemic anti-PAD4 antibody concentrations.

Anti-PAD4 antibody levels correlate with ACPA and nucleosome autoantibody levels in CF serum

Even if ACPA and anti-nucleosome autoantibody levels were not elevated in CF patients (Fig. 2), a correlation between them and anti-PAD4 antibody levels would reveal a stronger clinical link between the production of these autoantibodies in CF. Therefore, we correlated anti-PAD4 antibody levels with serum ACPA or anti-nucleosome autoantibody levels in CF patients or the control, non-CF cohorts. As data presented in Figure 5 show, significant correlations were found between anti-PAD4 antibodies and ACPAs, and anti-PAD4 and anti-nucleosome autoantibodies in CF sera. The same pairwise comparisons did not yield any significant correlations in control donors: 1) anti-PAD4 antibody vs. ACPA, r=0.1271, p=0.5634; 2) anti-PAD4 antibody vs. nucleosome autoantibody, r=0.0269, p=0.9028.

Conclusions

Lung disease characterized by bacterial infections and neutrophilic inflammation remains the major complication responsible for CF disease morbidity and mortality. The primary mechanism by which neutrophils are thought to cause lung disease is via release of their DNA and granule content: NE-mediated proteolytic damage and MPO-mediated oxidative stress. Long-term consequences of neutrophil dysfunction in CF airways must also be considered. Chronic extracellular presence of neutrophil components could break immune tolerance and trigger an autoimmune response further contributing to the disease. NETs represents a potential mechanism of neutrophil-mediated lung damage in CF since they have been identified as the source of autoantigens in well-known autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis and psoriasis (33–37). Our main goal was to explore whether
autoantibodies directed against NET components are detectable in the blood of CF patients and correlate with lung disease.

Elevated levels of MPO were found in the blood of CF patients compared to healthy volunteers and correlated with the degree of airway disease. This is consistent with previous findings of other reports and proposes that serum MPO could be a potential systemic biomarker for CF lung disease (38). Our results are also in line with previous observations by other groups detecting higher blood concentrations of MPO in RA and SLE patients (39, 40). Although elevated levels of NE or NE-antitrypsin complexes were previously found to be associated with CF lung disease (41, 42), CF patients in our study did not show significantly higher levels of NE/antitrypsin complexes compared to healthy controls (p=0.0627) but their significant correlation with the degree of CF airway obstruction suggests their potential physiological relevance. The reason for increased blood levels of these primary granule components could be their higher cellular release from blood neutrophils or a larger neutrophil output of the bone marrow in CF patients. Irrespective of their origin, elevated systemic levels of these inflammatory markers indicate ongoing inflammation in CF patients.

Levels of cell-free double-stranded DNA and NETs defined as complexes of NE and DNA were not elevated in CF serum. These results were somewhat surprising since other studies done on patients with bacterial infections or autoimmune diseases detected serum NET levels higher than in control patients. In a collaborative project we found that NET levels (MPO-DNA) in human plasma are elevated in patients with active tuberculosis compared to healthy controls or patients with latent TB (43). NET levels changed in parallel with serum MPO and NE levels and decreased upon antibiotics treatment of TB (43). Elevated serum NET levels (MPO-DNA complexes) were detected in children with meningococcal sepsis that, however, did not correlate with serum cell-free DNA levels (44). Increased blood levels of NETs (MPO-DNA) have been found in RA patients (45). Enhanced levels of NETs (MPO-DNA complexes) were observed in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) patients but they did not correlate with serum cell-free DNA levels and disease severity (46). On the other hand, elevated serum NET (MPO-DNA) levels were found in pregnant women that correlated with serum cell-free DNA concentrations indicating that most circulating DNA derives from NETs in pregnancy (47). In summary, recent studies demonstrate that a correlation between blood cell-free DNA and NETs levels is not obvious and most likely depends on the circumstances, each disease representing a unique case to be looked at individually. Despite the abundance of extracellular DNA and NETs in CF airways, we failed to detect their elevated levels in CF blood. Nonetheless, our results presented here are the first to quantitate and show feasibility to measure NET levels (NE-DNA), not simply DNA concentrations, in any clinical sample obtained from CF patients.

Although ACPA levels in CF serum were slightly elevated compared to controls, the difference remained non-significant. Similar to a recent study (21), our work also reports that circulating ACPA levels are significantly higher in RA than in CF patients. CF patients do not seem to develop a general autoimmune response to citrullinated proteins. Despite
their robust presence in CF airways, immune tolerance against citrullinated proteins is likely kept and prevents activation/formation of autoreactive B cells.

Nucleosome autoantibodies are characteristic hallmarks of SLE patients (48) and their formation has been linked to NET formation (49). In CF, we did not detect elevated levels of nucleosome IgG antibodies in the blood compared to healthy controls arguing against a systemic immune response to extracelluar chromatin. As expected, the SLE cohort in our study also presented enhanced levels of nucleosome autoantibodies. The absence of chromatin autoantibodies in CF could result from DNAse-mediated digestion of NETs/nucleosomes in the airways or NE/MPO-mediated damage of their epitopes crucial to elicit an immune response.

PAD4 has been shown to be expressed in cells of various haematopoietic lineages including neutrophils, eosinophils, monocytes, lymphocytes and NK cells (50–52). PAD4 is detectable in circulating monocytes and synovial macrophages of RA patients (50). PAD4 has been described in several cancers, as well (53, 54). Given the abundance of neutrophils in CF airways, high PAD4 expression in neutrophils and the central role of PAD4 as the main mediator of NET release, neutrophils are the most likely source of PAD4 in CF (55). When neutrophils extrude NETs, PAD4 is also released into the extracellular space (56). Therefore, an enzyme that is always located intracellularly in neutrophils will be exposed to the immune system following NET formation, suggesting PAD4 is an ideal neoantigen candidate. Indeed, anti-PAD4 antibodies are detected and well-characterized in RA (57–61). Anti-PAD4 antibodies correlate with ACPA levels, longer disease duration and shared epitope alleles, but not with serum PAD4 concentration in RA (58, 62, 63). Anti-PAD4 antibodies can be detected in the preclinical phase and precede the clinical onset of the disease in RA patients (64). Whether anti-PAD4 antibodies contribute to RA disease pathogenesis and by what mechanism, remains unclear. Interestingly, a subset of RA patients have antibodies against both PAD isoforms, PAD3 and PAD4 (65). These anti-PAD3/4 cross-reactive antibodies have been linked to RA-associated interstitial lung disease and more erosive arthritis, and they lower the Ca$^{2+}$-binding threshold required for PAD4 activation, an effect potentially relevant to the pathogenesis of RA-associated interstitial lung disease (24, 65, 66).

We found that anti-PAD4 antibodies are significantly elevated in CF patients compared to control subjects. This report is the first to document this finding and adds a new antibody to the growing list of autoantibodies detected in CF. Interestingly, CF patients have elevated anti-PAD4 antibody, but not ACPA, levels while RA is characterized by increased concentrations of both autoantibodies. The reasons for the different autoantibody pattern between RA and CF remains to be determined. Although not elevated in CF, ACPA and anti-nucleosome IgG levels also correlated with anti-PAD4 antibody concentrations. No such correlation was observed in control subjects. These results suggest a mechanism that delivers nucleosomes and enzymatically active PAD4 at the same time to trigger an autoimmune response. NET formation is a primary suspect since neutrophils expel PAD4 and chromatin during the process (9, 56). Furthermore, the correlation between anti-PAD4 antibody levels and CF lung disease indicates the potential clinical relevance of the chronic extracellular presence of PAD4 in CF disease pathogenesis. Anti-PAD4 antibodies reported here are the
first NET-specific marker in CF that has been linked quantitatively to lung disease. Anti-PAD4 antibodies also represent a new candidate for CF biomarkers. Systemic biomarkers are favored over biomarkers detectable in airway fluids because blood samples can always be obtained whereas many CF patients, particularly CF children and teens, are unable to expectorate sputum. It also remains to be investigated whether these antibodies are more than just potential new biomarkers and cause any of the clinical symptoms in CF. Autoantibodies could drive relatively infrequent autoimmune-like symptoms of unknown etiology in CF or, more importantly, they could contribute to lung disease. Autoimmunity is thought to play a role in lung diseases seen in RA, SLE, and ANCA-positive vasculitis. However, the site of lung disease in these disorders is the interstitial space in RA and SLE and the pulmonary microvasculature in ANCA-positive vasculitis but not in the airways as is the case in CF. Indeed, there was little appreciation for a role of autoimmunity as a driver of airway disease until the discovery of mucosal-associated invariant T (MAIT) cells (67). MAIT cells are a subset of T lymphocytes that are abundant in human airways and whose ligands include bacterial and mycobacterial metabolites (67). Recent work in asthma and COPD is raising the possibility of MAIT cells playing a role in the development of these airway diseases (68). Our work here has shown that anti-PAD4 autoantibodies related to NET formation correlate with more severe airway disease in CF. Autoantibodies could contribute to CF lung disease either independently from neutrophils or in a collaborative effort with them. We acknowledge the limitations of this correlative study including relatively small sample size derived from one CF center and its cross-sectional nature. Larger patient cohorts available at multiple sites have to be analyzed longitudinally in the future to establish a stronger clinical relevance of anti-PAD4 autoantibodies in CF. Based on the data presented here, however, we are in a position to propose the exciting possibility that autoantibody-mediated neutrophil activation provides a new potential mechanism by which autoantibodies and neutrophils together could drive CF lung disease.

CF is a complex disease complicated by genetic, environmental, microbiological and immunological factors. To learn about the clinical importance of anti-PAD4 antibodies in CF, we divided our CF cohort according to available clinical information and comorbidities. There was a significant association of anti-PAD4 antibody levels only with *P. aeruginosa* airway infection. Similarly, anti-BPI IgG levels were reported to correlate with airflow obstruction in CF and with presence of mucoid *P. aeruginosa* (21, 69). Anti-carbamylated peptide IgG antibodies derived from NETs have also been detected in CF but were found not to be associated with *P. aeruginosa* infection (21). Our results indicate that anti-PAD4 antibodies are more similar to BPI antibodies in CF in terms of clinical associations and therefore represent another, potential consequence of *P. aeruginosa*-induced NET formation, a mechanism we have studied for years (10, 11, 70, 71).

In summary, our novel findings reported here on elevated anti-PAD4 antibody levels in the blood of CF patients correlating with airflow obstruction adds a new puzzle to the growing field of autoimmunity in airways diseases and highlights the potential relevance of the interplay between infection, inflammation and autoimmunity in CF (72, 73).
Acknowledgements

Human CF patient samples were provided by the CF Biospecimen Registry at the Children’s Healthcare of Atlanta and Emory University CF Discovery Core. We would like to thank Jane Wei and Julie Flores, data managers of the Biospecimen Registry, for retrieving and verifying the clinical data for the CF subjects enrolled in this study.

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Abbreviations

- **CF**: cystic fibrosis
- **PAD4**: peptidylarginine deiminase 4
- **ACPA**: anti-citrullinated protein antibody
- **SLE**: systemic lupus erythematosus
- **RA**: rheumatoid arthritis
- **NET**: neutrophil extracellular traps
- **NE**: neutrophil elastase

References


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Highlights

- CF serum contains elevated levels of anti-PAD4 antibodies
- Anti-PAD4 antibody levels in CF serum correlate with lung disease
- Anti-PAD4 antibody levels in CF are associated with *Pseudomonas aeruginosa* lung infection
- Levels of NETs, cell-free DNA, anti-nucleosome antibodies or ACPAs are not elevated in CF
- Pattern of autoantibodies in CF is different from that seen in two diseases where autoantibody formation is linked to pathogenesis – rheumatoid arthritis and systemic lupus erythematosus.
Figure 1. Serum MPO concentrations are elevated in CF while levels of cell-free DNA, NETs and NE are not.

A) Serum levels of MPO measured by ELISA are elevated in CF compared to control subjects. B) Serum NE levels determined by ELISA are not significantly elevated in sera of CF patients. C) Serum MPO levels negatively correlate with lung function of CF patients. D) Serum levels of MPO and NE positively correlate with each other. E) Serum levels of cell-free, double stranded DNA measured by PicoGreen DNA kit are not different between CF patients and control individuals. F) Serum levels of NETs defined as NE-DNA complexes measured by ELISA are not different in CF from control subjects. Sera from 37 CF patients and 23 healthy controls were compared. Significance of pairwise comparisons were performed with Mann-Whitney test while correlations of two variables were determined by Spearman correlation analysis via calculation of correlation coefficient (r). Each dot represents a separate human subject. Ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001. MPO, myeloperoxidase; NE, neutrophil elastase; HC, healthy controls; CF, cystic fibrosis; FEV_{1% pred} forced expiratory volume in 1 per cent predicted.
Figure 2. CF patients do not have elevated serum levels of anti-citrullinated protein or anti-nucleosome autoantibodies.

Concentrations of A) ACPAs and B) anti-nucleosome autoantibodies were quantitated by ELISA in sera of CF, RA, SLE patients and healthy subjects. Each dot represents a separate human subject. Results were compared for significance among cohorts by Kruskal-Wallis test and Dunn’s multiple comparison. Ns, not significant; *, p<0.05, **, p<0.01, ***, p<0.001. CF, cystic fibrosis; HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; ACPA, anti-citrullinated protein antibody.
Figure 3. CF patients have elevated systemic levels of anti-PAD4 antibodies that correlate with airflow obstruction.

A) Concentrations of anti-PAD4 antibodies were determined by ELISA in sera of CF (n=37), RA (n=20), SLE patients (n=21) and healthy subjects (n=23). Results were compared by Mann-Whitney test. B) Serum anti-PAD4 antibody levels of CF patients negatively correlate with lung function measured as FEV$_{1\%\text{pred}}$ (Spearman correlation coefficient, r). Each dot represents a separate human subject. **, p<0.01; ***, p<0.001. HC, healthy controls; CF, cystic fibrosis; FEV$_{1\%\text{pred}}$, forced expiratory volume in 1 per cent predicted; PAD4, peptidylarginine deiminase 4; Ab, antibody; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.
Figure 4. Elevated serum anti-PAD4 antibody levels in CF are associated with *P. aeruginosa* lung infection.

Anti-PAD4 antibody levels measured in sera of CF patients in Figure 3 were associated with the presence of the dominant bacterial respiratory pathogens. A) Serum concentrations of anti-PAD4 antibodies are higher in CF patients with *P. aeruginosa* than in CF patients without *P. aeruginosa* or in control subjects. B) Although it is not significant (p=0.0547), there is a trend of worse lung function of CF patients with *P. aeruginosa* infection compared to CF patients without *P. aeruginosa* in our cohort. C) Anti-PAD4 antibody levels in CF patients with *P. aeruginosa* were compared according to the mucoid status of the bacterium. Anti-PAD4 antibodies in the blood of CF patients are not related to the presence of D) *Staphylococcus aureus*, E) MRSA or MSSA, or F) *P. aeruginosa* +/- MRSA +/-MSSA. 37 CF patients were analyzed. Each dot represents a separate human subject. Mann-Whitney test was used for pairwise comparisons while Kruskal-Wallis test was used to compare more than two cohorts. Ns, not significant; *, p<0.05. CF, cystic fibrosis; FEV\(_1\) \%pred forced
expiratory volume in 1 per cent predicted; HC, healthy control; PAD4, peptidylarginine deiminase 4; SA, *Staphylococcus aureus*; PA, *Pseudomonas aeruginosa*; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*.
Figure 5. Anti-PAD4 autoantibody levels correlate with ACPA and anti-nucleosome autoantibody levels in CF serum.

Levels of anti-PAD4 antibodies measured in sera of CF patients were correlated with serum concentrations of A) ACPAs and B) anti-nucleosome antibodies. Sera from 37 CF patients were tested. Each dot represents a separate CF patient. Spearman correlation analysis and coefficient (r). *, p<0.05. CF, cystic fibrosis; PAD4, peptidylarginine deiminase 4; Ab, antibody; ACPA, anti-citrullinated protein antibody.
Table 1.

Sex and age distribution of patient cohorts.

<table>
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<tr>
<th>Patient cohorts</th>
<th>Cystic Fibrosis (CF)</th>
<th>Healthy controls (HC)</th>
<th>Rheumatoid arthritis (RA)</th>
<th>Systemic Lupus Erythematosus (SLE)</th>
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<td>29.2±11.6</td>
<td>32.5±4.8</td>
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Table 2.

Additional clinical characteristics of CF patients.

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<tr>
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<tbody>
<tr>
<td>Without <em>P. aeruginosa</em> infection</td>
<td>14</td>
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<tr>
<td>With <em>P. aeruginosa</em> infection</td>
<td>23</td>
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<tr>
<td>Mucoid</td>
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</tr>
<tr>
<td>Not reported</td>
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<td>(5.4%)</td>
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<tr>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>16</td>
<td>(43.3%)</td>
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