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Strong toll-like receptor responses in cystic fibrosis patients are associated with higher lung function ^{☆,☆☆}

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Abstract

Background: Cystic fibrosis (CF) airways disease varies widely among patients with identical cystic fibrosis transmembrane conductance regulator (*CFTR*) genotypes. Robust airway inflammation is thought to be deleterious in CF; inter-individual variation in Toll-like receptor (TLR)-mediated innate immune inflammatory responses (TMIIR) might account for a portion of the phenotypic variation. We tested if TMIIR in people with CF are different than those of healthy controls, and whether higher TMIIR in people with CF are associated with reduced lung function.

Methods: We cultured whole blood from clinically stable subjects with CF ($n = 76$) and healthy controls ($n = 45$) with TLR agonists, and measured cytokine production and expression of TLR-associated genes. We tested for differences in TLR-stimulated cytokine levels between subjects with CF and healthy subjects, and for associations between cytokine and gene expression levels with baseline lung function (forced expiratory volume in one second percent predicted (FEV₁ %)) and decline in FEV₁ % over time.

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Author Contributions: M.M.W had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. M.L.A., C.H.G., and M.M.W conceived and designed the experiments. M.L.A. and P.A.C recruited the study subjects. V.D., C.N., T.D.H., and F.R. performed the experiments. S.K., C.H.G., and M.M.W performed statistical analyses. S.K., K.B.H., C.H.G., M.L.A., and M.M.W wrote the manuscript. All authors read and approved the submission of the manuscript.

¹These authors contributed equally.

Declaration of Competing Interest

The authors report no conflicts of interest.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2019.11.009.

Results: TMIIR in blood from subjects with CF were lower than in healthy controls. Expression of TLR regulators *SARM1*, *TOLLIP*, and *AKT1* were downregulated in CF. In subjects with CF we found that lower TLR4-agonist-induced IL-8 was associated with lower FEV₁ % at enrollment ($p < 0.001$) and with greater five year FEV₁ % decline ($p < 0.001$).

Conclusions: TMIIR were lower in people with CF relative to healthy controls; however, unexpectedly, greater whole blood TMIIR were positively associated with lung function in people with CF. These findings suggest a complex interaction between inflammation and disease in people with CF.

Keywords

Cystic fibrosis; Toll-like receptors; Respiratory function tests; Innate Immunity

1. Introduction

Cystic Fibrosis pulmonary disease is characterized by chronic airway infections and concomitant non-resolving inflammation that usually leads to respiratory failure within the fourth decade of life. The disease results from patients bearing two mutant copies of the cystic fibrosis transmembrane conductance regulator (*CFTR*), a channel that transports chloride and bicarbonate [1]. Over 2000 different mutations in *CFTR* have been identified, and these mutations are associated with varying severity of disease based on how much residual *CFTR* activity remains. Although the exact mechanisms by which a lack of *CFTR* activity results in disease remain unclear, patients with CF develop thick airway mucus, dysfunction of mucociliary clearance and other host airway defense mechanisms, and are subsequently prone to colonization with a characteristic panel of bacterial and fungal pathogens, including *Staphylococcus aureus*, and the opportunistic pathogens *Pseudomonas aeruginosa*, *Burkholderia species*, and *Aspergillus fumigatus*.

Studies have demonstrated associations between increased airway inflammation in CF and more rapid decline in lung function [2]. Remarkably, patients with the same *CFTR* genotype manifest high inter-individual variation in airway inflammation and divergent airway disease progression [3]. This observation has led to the hypothesis that additional genetic loci likely contribute to pathophysiology of CF disease. Several studies have identified polymorphisms in genes related to innate immune function that appear to alter CF disease severity, including *MUC4/MUC20*, *SLC9A3*, *SLC26A9*, *HLA Class II*, *AGTR2/SLC6A14*, *IFRD1*, *DCTN4*, and *EHF/APIP* [4].

Toll like receptors (TLRs) are a critical component of the innate immune system. These transmembrane receptors are expressed on a wide variety of cells, and are involved in the recognition and response to a broad range of microbial pathogens. There are more than 10 different TLRs that each recognize specific pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by TLRs initiates an intracellular signaling cascade leading to cell activation and inflammatory mediator production [5]. Published studies describe potentially opposing roles for TLR-mediated activation in the context of CF pulmonary disease. In mice, TLR2, TLR4, TLR5, and TLR signaling molecule MyD88 are essential for host defense against *P. aeruginosa* [6,7]. MyD88 knock-out mice, however, are

protected from fatal pneumonia caused by *Burkholderia cenocepacia* [8]. Macrophages from CFTR^{-/-} mice mount hyper-inflammatory responses to the TLR4 agonist LPS compared to cells from healthy mice [9]. In patients with CF, the bronchiolar inflammatory response to infection is higher than in patients with airway disease not due to CF [10]. However, TLR-mediated inflammatory cytokine production in human peripheral monocytes *ex vivo* is reduced in patients with CF relative to healthy controls [11]. One proposed explanation for this finding is that leukocytes are repeatedly exposed to lipopolysaccharide (LPS) chronically translocating into the circulation of CF patients leading an LPS-tolerant state [12]. The primary goal of this study was to determine whether TLR-mediated *ex-vivo* responses were associated with basal lung function or clinically significant lung function decline in people with CF. We hypothesized that more robust TLR-mediated inflammatory responses would be associated with worse lung function.

2. Materials and methods

2.1. Study subjects

Patients with CF aged 18 years or older were recruited from University of Washington Medical Center (UWMC), Seattle, WA. These studies were done in accordance with the Declaration of Helsinki and were approved by the Institutional Review Board at the University of Washington under study protocol #26059. (See the online supplement).

2.2. Ex vivo whole blood assay

Ex vivo whole blood assays were performed using previously published methods with a panel of stimulants for TLR2/6, TLR2/1, TLR5, TLR7, and TLR4 [13]. (See the online supplement).

2.3. Gene expression measurements

Gene expression was measured using TaqMan assays (Thermo Fisher Scientific, Waltham, MA). Relative expression levels were determined using the Ct method [14]. (See the online supplement).

2.4. Statistical analyses

Differences between PAMP-induced monocyte count-normalized cytokine levels in healthy donors and CF patients were compared using Student's *t*-test with correction for multiple hypotheses testing using a false discovery rate (FDR) [15]. We determined the relationship between baseline FEV₁ % predicted and cytokine levels using multiple linear regression. We identified associations between cytokine responses and rate of decline in FEV₁ % over the five year period prior to blood sampling with general estimating equation (GEE) models using robust errors. Both models included adjustments for age and gender. Difference in gene expression between healthy donors and CF patients was analyzed with Student's *t*-tests. We tested for associations between gene expression levels and baseline FEV₁ % using linear regression with robust errors. We also adjusted the regression analyses for age and gender only, or age, gender, and expected CF disease severity based on genotypes for severe (*CFTR* mutation classes I-III) and mild (classes IV-V). Software used included STATA 14

(StataCorp, College Station, TX), Golden Helix SVS (Golden Helix, Inc., Bozeman, MT) and GraphPad Prism 7 (GraphPad, La Jolla, CA).

3. Results

3.1. Subject demographics

We enrolled clinically stable subjects with CF ($n = 76$) and healthy controls ($n = 45$) matched for age. CF subjects were predominantly male (58%), had a mean age of 28.5 years, and the vast majority were carriers of the F508del *CFTR* allele (55.3% homozygous, 39.5% heterozygous, 5.3% non-F508del. Table 1 and e-Table 2). Lung function in subjects with CF on enrollment was moderately reduced (mean FEV₁ % 62.7 (95% CI: 56.8, 68.7)) and the mean rate of decline in FEV₁ over the five years prior to enrollment was -1.4% per year (95% CI: -2.01, -0.74). Baseline FEV₁ % was not significantly different between subjects with a mild vs severe *CFTR* allele classification (e-Figure 1). Nearly all subjects (90%) had pancreatic insufficiency. The majority (66%) were colonized with *P. aeruginosa*. Only one subject was colonized with *B. multivorans*. Healthy controls were well matched for age (mean age = 30 years) but had a slightly lower proportion of males (44%). Mean blood monocyte counts were lower in healthy controls (0.35×10^3 cells/ μ L) relative to people with CF (0.62×10^3 cells/ μ L).

3.2. Magnitude of TMIIR in whole blood is reduced in people with CF relative to healthy controls

We compared TLR agonist-induced cytokine and chemokine responses in *ex vivo* whole blood from people with CF and healthy controls as previously described [16]. Cytokine and chemokine levels were low in supernatants from unstimulated blood (e-Table 2). Monocyte-normalized levels of IL1RA ($p = 0.009$) and IL-6 ($p < 0.0001$) were significantly higher and lower, respectively, in supernatants from people with CF than from healthy controls (e-Table 2). There were no significant differences in unstimulated levels of TNF α , IL-8, or MCP-1 between people with CF and healthy controls. A subset of the TLR agonist-induced pro-inflammatory cytokines and chemokines were lower in samples from people with CF than healthy controls. Significant differences are shown in bold in e-Table 2, and graphed in e-Figure 2. Most notably, we observed lower production of IL-6 and TNF α by CF cells in response to TLR4 and TLR5 agonists. Lower levels of monocyte normalized mediator concentrations were seen in samples stimulated with *Yersinia pestis* (YP) LPS ($p < 0.0001$) and flagellin ($p = 0.006$). In contrast, we observed higher levels of the anti-inflammatory cytokine IL1RA in response to FSL (TLR2/6)-stimulation by CF cells than cells from healthy controls ($p = 0.017$).

3.3. Higher TLR agonist-induced cytokine/chemokine production associated with better lung function on enrollment

We then tested for associations between TLR agonist-induced cytokine/chemokine production levels and FEV₁ % on enrollment in the CF cohort. We found that higher TLR agonist-induced cytokine/chemokine production was associated with better lung function on enrollment. For instance, every unit change in log10-transformed monocyte normalized flagellin-induced IL-8 was associated with over a 30% change in FEV₁ % ($\beta = 30.6$, 98%

CI [20.9, 40.3], $p < 0.001$). Associations with $p < 0.05$ are shown in Table 2. Similar associations were observed across multiple stimulus/cytokine combinations, but PAMP-induced IL-8 levels exhibited the strongest and most consistent associations with baseline lung function (Fig. 1). IL-8 production after TLR agonist stimulation was significant for nearly all agonists after multiple correction with Bonferroni $p < 0.00125$ for 40 tests with $\alpha = 0.05$. Adjustments for age, gender, and *CFTR* allele classification (mild/severe) did not change these associations.

3.4. Downregulation of TLR signaling pathways in CF

Given the lower PAMP-induced responses in CF subjects relative to healthy controls we sought to determine if expression of genes associated with regulation of TLR signaling were differentially expressed in blood from CF subjects relative to healthy controls. We performed qRT-PCR to measure levels of mRNA transcripts for selected TLR-related genes (Fig. 2, e-Table 3). We found lower expression of *SARM1* (Sterile-alpha and Armadillo motif containing protein 1 [17]), *TOLLIP* (Toll-Interacting Protein [18]), and Phosphoinositide 3-kinases (PI3K) signaling molecule *AKT1* [19] in CF patient blood compared to healthy individuals.

3.5. Higher TLR agonist-induced cytokine/chemokine production is associated with lower five year decline in lung function

TLR agonist-induced IL-8 levels were most consistently associated with lung function on enrollment. Therefore, we sought to determine whether TLR agonist induced IL-8 levels were also associated with the rate of decline in lung function over the five years prior to enrollment in the subset of patients for whom these data were available ($n = 69$). The overall FEV₁ % decline was 1.4% per year, which is representative of the general CF population [20]. We found that TLR4 and TLR5 agonist-induced IL-8 levels were inversely related to lung function decline over 5 years. Each unit increase in log10-transformed flagellin-induced IL-8 was associated with a 1.2% improvement in FEV₁ % decline per year from the average annual decline of -1.4% (CI [-2%, -0.7%] over the 5 years (Table 3).

3.6. Expression of TLR pathway genes is associated with baseline FEV₁ % in CF patients

We examined the relationship between the expression of the TLR signaling-associated genes in blood and lung function on enrollment. Higher expression of TLR pathway regulators, including *SARM1* ($p < 0.001$), *TOLLIP* ($p = 0.001$), *INPP5D/SHIP* ($p = 0.006$), and *AKT1* ($p = 0.008$) were strongly positively associated with higher baseline FEV₁ %. Additionally, *TLR4*, *TREMI*, *IRAKM*, and *TLR7* expression was positively associated with better baseline FEV₁%, but all had p -values > 0.01 (e-Table 4, e-Figure e-Table 5).

4. Discussion

Studies have shown that increased levels of inflammatory markers in sputum of people with CF correlate with lower lung function and predict faster lung function decline [21]. The establishment of airway colonization with gram negative bacteria, particularly *Pseudomonas aeruginosa*, also correlates with increased morbidity and mortality in people with CF [22]. In addition to the continued presence of bacteria in the CF airway, which can trigger persistent

inflammation, CF immune cells have been found *in vitro* to mount heightened immune cell inflammatory responses. However, circulating monocytes in people with CF exhibit a “tolerant” phenotype, in which cells are less responsive to stimuli, such as LPS, as compared to cells from healthy donors, possibly due to the leak of LPS from the chronically colonized lung into the circulation [11,12]. The contributions of abnormal innate immune responses in CF to acquisition of chronic airway infections and to non-resolving, pathologic airway inflammation in human subjects requires further elucidation.

CF animal models have shed light on the roles of individual components of the immune system in CF pathophysiology, but generating cohesive results has been difficult. While there have been excellent studies in mice lacking CFTR that have examined inflammatory responses, the implications of these studies is unclear given that mice do not recapitulate the human CF phenotype of chronic airway infections [23]. In a porcine model, lungs of the *CFTR*^{-/-} pigs appear normal at birth with minimal lung inflammation that does not differ from wild-type controls [24], but they have hyper-inflammatory macrophages at birth [25]. In the CF ferret model, neonatal animals deficient in CFTR show deficiencies in IL-1 β levels but not IL-8 or TNF α , and a deficiency in clearance of *Pseudomonas* but not *Staphylococcus*, and lung disease without infection [26]. These data together demonstrate an immunological dysregulation in animals with CF, but do not clarify how abnormal innate immune responses contribute to morbidity associated with chronic airway inflammation in CF.

We sought to determine if peripheral innate immune responses in adult subjects with CF correlate with degree of lung damage and/or rate of lung function decline. We hypothesized that patients with stronger circulating blood inflammatory responses would have lower FEV₁ %, and that magnitude of TLR responses *ex vivo* would predict more rapid lung function decline in people with CF. To investigate this hypothesis, we used an *ex vivo* whole blood TLR stimulation protocol to study TLR responses in patients with CF and healthy controls [13,16]. Contrary to our hypothesis, we found that increased innate immune responses in whole blood were associated with attenuated decline in FEV₁ % in adult patients with CF over five years. Patients with baseline FEV₁ % > 70% had consistently more cytokine production in whole blood upon stimulation with TLR ligands than those with lower FEV₁ % (Fig. 1). We also analyzed expression of genes (*SARM1*, *TOLLIP*, *INPP5D/SHIP*, and *AKT1*) whose products are known to modulate TLR-agonist. Expression of TLR-regulatory genes was downregulated in people with CF compared to healthy controls (Fig. 2). Increased expression of these genes associated positively with lung function at baseline in CF samples (e-Figure 3).

These results suggest that there is a general suppression of peripheral blood leukocyte responses in people with CF, both the pro-inflammatory TLR responses and the activity of TLR-negative regulatory proteins, and that less immune cell suppression correlates with better lung function. Our data do not distinguish whether this differential immune responsiveness between subjects with CF and healthy donors is due to a direct, inherent effect of CFTR deficiency on peripheral blood immune cells, or an indirect/secondary effect of chronic lung infection in people with CF. However, recent studies found that addition of plasma from people with CF to peripheral blood mononuclear cells from healthy donors [27]

led to suppression of inflammatory gene expression, supporting the idea that immunomodulation may be a secondary effect.

The existence of damaging excessive inflammation in CF has been a predominant paradigm and has led to several interventional trials directed at attenuation of inflammation. Both corticosteroids and ibuprofen have shown benefit in terms of improved lung function in clinical trials [28,29]. Our data suggest that the interaction of inflammation with lung damage and airflow obstruction may be more complex, and that some inflammatory responses may be protective to lung function. Deficiencies in innate immunity responses could impair initial clearance of airway pathogens such as *S. aureus* or *P. aeruginosa* that then leads to the neutrophilic inflammation seen in the bronchiectatic CF lung, and could also permit more rapid bacterial replication, which could lead to more frequent exacerbations and therefore faster decline in lung function. However, there are other explanations for the correlation between more robust TLR responses and more preserved lung function in people with CF. Individuals with higher levels of lung inflammation and lung damage, who are predisposed to faster lung function decline, may leak more LPS and bacterial products into the bloodstream, thus causing a more profound tolerance phenotype, with less robust leukocyte TLR-responses. Future studies will need to examine cell-specific innate immune responses early in CF, and follow these responses longitudinally, to better understand whether impaired innate immune responses are a marker or mediator of lung function decline.

This study has several important limitations. First, the cohort size was modest. Second, we assayed a single time point for the measurement of the innate immune response. Third, our study design permitted only a retrospective assessment of clinical phenotype. Fourth, the use of whole blood enabled high-throughput evaluations of responses to multiple antigens from a large number of subjects, but prevented us from ascertaining which cell populations are responsible for the phenotypes we observed. Finally, FEV₁ measures lung pathology, and our study measured TLR responses in peripheral blood cells, and not tissue specific leukocytes. However, peripheral blood cells are relevant for at least two key reasons: [1] studies suggest that in CF and other chronic airways diseases, cells are recruited to the airways from the systemic circulation [30,31], and [2] peripheral blood cells are more readily obtained than tissue leukocytes, and thus they have a greater use as prognostic or diagnostic biomarkers.

4.1. Summary

We have shown that inflammatory responses to TLR agonists measured *ex vivo* in whole blood are lower in people with CF compared to healthy controls. We have also shown that higher responses to TLR4 and TLR5 agonists are associated with greater baseline lung function (FEV₁ %) and slower lung function decline in people with CF. Our retrospective study design does not allow for assignment of causality; however, this unexpected finding raises the possibility that TLR-mediated inflammatory responses are important for maintenance of lung function in CF, and that suppression of specific innate immune responses may contribute to lung function decline in CF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
FDR	false discovery rate
FEV₁ %	forced expiratory volume in one second percent predicted
GEE	general estimating equation
LPS	lipopolysaccharide
PAMPs	pathogen-associated molecular patterns
SARM1	sterile-alpha and Armadillo motif containing protein 1
TLR	toll-like receptor
TMIIR	TLR-mediated innate immune inflammatory responses
TOLLIP1	toll-Interacting Protein
YP	<i>Yersinia pestis</i>
IQR	interquartile range
CI	confidence interval

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Fig. 1. Association of higher induced IL-8 levels with better baseline FEV₁ %.

Graphical representation of relationship of IL-8 levels upon TLR ligand stimulation with baseline FEV₁ %. Tertiles of lung function were calculated as 1) FEV₁ % below 40%, 2) FEV₁ % 40% to 70%, and 3) FEV₁ % above 70%. The plots show median and the interquartile range with 10–90 percentile whiskers.

Fig. 2. Comparison of monocyte count-normalized TLR and inflammatory gene expression in whole blood of CF patients and healthy donors.

RNA isolated from whole blood was subjected to qRT-PCR using TaqMan chemistry. Shown are significantly differentially expressed genes with fold differences normalized to GAPDH, control RNA, and monocyte count. Median, intra-quartile range, 5 and 95th percentiles are shown. T-test results are shown in e-Table 3. White boxes: healthy donors; black boxes: CF patients.

Table 1

Subject characteristics.

Basic Demographics	CF (n = 76)	Healthy (n = 45)
% Female (% , n)	42% (32)	56% (25)
Age (median, IQR)	28.5 (25, 36)	30 (25, 41.5)
Monocytes (x10 ³ cell/ μ L, median, IQR))	0.62 (0.48, 0.81)	0.35 (0.29, 0.47)
FEV₁ % Calculations		
FEV ₁ % Predicted, (mean, 95% CI)	62.7 (56.8, 68.7)	
FEV ₁ % Predicted 5 year rate (mean, 95% CI)	-1.4, (-2.01, -0.74)	
CF Clinical Parameters (% , n)		
Pancreatic Enzyme Use	90% (69)	
<i>B. multivorans</i> culture positive	1% (1)	
<i>Pseudomonas aeruginosa</i> positive	66% (50)	
CFTR Genotypes (% , n)		
F508del Homozygous	55.3% (42)	
F508del Heterozygous	39.5% (30)	
Non-F508del	5.3% (4)	
CFTR classes I-III only (severe)	84.2% (64)	
One CFTR class IV-V allele (mild)	14.5% (11)	
F508del with uncharacterized second allele	1.3% (1)	

Table 2Association of baseline FEV₁ % predicted with induced cytokine levels.

TLR	Stimulus (dose)	Cytokine	Coefficient (95% CI)	p-value
NA		IL-6	20 (1.9, 38.1)	<i>p</i> = 0.031
		IL-8	21.7 (8, 35.4)	<i>p</i> = 0.002
		MCP-1	17.4 (7, 27.8)	<i>p</i> = 0.001
		TNF-α	23 (3.5, 42.4)	<i>p</i> = 0.021
TLR1/2	Pam3CSK4	IL-8	7.7 (1.5, 13.9)	<i>p</i> = 0.015
TLR2/6	FSL (100 ng/mL)	IL-8	24.4 (9, 39.8)	<i>p</i> = 0.002
TLR4	O111:B4 (1 ng/mL)	IL-6	12.5 (3.7, 21.4)	<i>p</i> = 0.006
		IL-8	24.3 (14.8, 33.9)	<i>p</i> < 0.001
		MCP1	16.2 (2.4, 30.1)	<i>p</i> = 0.022
		TNFα	13.6 (4.7, 22.6)	<i>p</i> = 0.003
	PaHexa (0.1 ng/mL)	IL1RA	18.2 (1.7, 34.8)	<i>p</i> = 0.031
		IL-6	9.6 (0.3, 18.8)	<i>p</i> = 0.042
		IL-8	27.1 (16.9, 37.3)	<i>p</i> < 0.001
		MCP-1	17.6 (2.2, 32.9)	<i>p</i> = 0.025
	Yp37 (1.0 ng/mL)	TNFα	13.3 (4.3, 22.3)	<i>p</i> = 0.004
		IL1RA	21.6 (2.7, 40.5)	<i>p</i> = 0.026
		IL-8	35.6 (23.8, 47.4)	<i>p</i> < 0.001
		MCP-1	21 (8.1, 33.9)	<i>p</i> = 0.002
TLR5	Flagellin (500 ng/mL)	TNF-α	43 (21.4, 64.7)	<i>p</i> < 0.001
		IL-8	30.6 (20.9, 40.3)	<i>p</i> < 0.001
		MCP-1	16.3 (0.6, 32)	<i>p</i> = 0.042
TLR7	R848 (1.0 mg/mL)	TNF-α	16.3 (0.6, 32)	<i>p</i> < 0.001
		IL1RA	26.8 (3.0, 50.6)	<i>p</i> = 0.028
		IL-8	32.6 (20.0, 45.2)	<i>p</i> < 0.001

Levels of whole blood monocyte-normalized cytokines were compared to baseline FEV₁ % in CF patients using linear regression with robust error calculation. The coefficients represent the change in baseline FEV₁ % associated with 1 unit change in the log10, monocyte-normalized cytokine or chemokine concentration. The base model was calculated with mean FEV₁ % predicted of 62.7% with 95% CI of 56.8% - 68.7%. Shown are results with *p* < 0.05. Boldfaced values passed Bonferroni correction for 40 tests.

Table 3

Association of FEV₁ % decline over 5 years with induced IL-8 levels.

TLR	Stimulus (dose)	Unadjusted		Adjusted (Age and Gender)	
		Coefficient (95% CI)	p-value	Coefficient (95% CI)	p-value
TLR4	O111:B4 (1 ng/mL)	1.45 (0.39 - 2.51)	$p = 0.007$	1.45 (0.4 - 2.49)	$p = 0.007$
	PaHexa (0.1 ng/mL)	1.79 (0.76 - 2.82)	$p < 0.001$	1.8 (0.78 - 2.39)	$p < 0.001$
TLR5	Flagellin (500 ng/mL)	1.25 (0.09 - 2.41)	$p = 0.035$	1.23 (0.07 - 0)	$p = 0.038$

GEE linear regression was performed on unit changes of cytokine levels against loss of FEV₁ %/year for all stimulus/cytokine combinations. The coefficients represent the decrease in the rate of FEV₁ % decrease per year associated with the log10 monocyte-normalized cytokine concentration for each subject. The mean annual FEV₁ % decline was -1.4% with 95% CI of -2% to -0.7%.