



Early View

Original research article

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Clinical implications of innate immune exhaustion in cystic fibrosis

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Take home message: Immune function is affected in CF, possibly altering disease progression. We report evidence of immune exhaustion, an immunomodulatory effect of intravenous antibiotic treatment, and a potential role of the Toll Like Receptor 3-pathway in CF lung disease.

Abstract:

Objectives: The lung disease progression in people with cystic fibrosis (pwCF) varies from one individual to another. Different immunological characteristics have been suggested to explain this variation, and we hypothesized that lung capacity may be associated with the innate immune response in pwCF. In an exploratory study, we aimed to investigate potential links between the innate immune response and lung function in pwCF using the standardized immune function assay TruCulture.

Methods: In a single-centre study with combined cross-sectional and longitudinal data before and after intravenous antibiotics, blood was sampled from *Pseudomonas aeruginosa* infected pwCF. Whole blood was analysed by TruCulture to reveal the unstimulated and stimulated cytokine release. Tobit regressions and Spearman's correlations were used to estimate the associations between lung function and cytokine release.

Results: We included 52 pwCF in the cross-sectional study and 24 in the longitudinal study. In the cross-sectional study, we found that compared to a healthy population the release of toll-like receptor (TLR)3, -4 and -7/8 stimulated IFN- γ , and IL-12p40 were reduced. Although TLR3-stimulated IL-1 β and IL-6 release increased with lung function, overall, cytokine release did not correlate well with lung function. In the longitudinal study, the cytokine release was modified by antibiotic treatment, but the cytokine release before antibiotic treatment did not associate with changes in lung function after treatment.

Conclusion: The stimulated cytokine release could not predict lung function levels or changes in pwCF, but our data indicate that pwCF experience exhaustion in the innate immune response after years of chronic bacterial infection.

Introduction

Persistent lung infection and inflammation remain the most significant challenge and cause of morbidity and mortality in cystic fibrosis (CF)[1]. Although the treatment of CF airway infections has improved for decades[2], the life expectancy among people with CF (pwCF) is still far from the expectancy in the general population[3]. For poorly understood reasons, some pwCF show a steep lung function decline at a very early age[4], while others have a normal lung function for decades[5]. Although some pwCF maintain a stable lung function over time, multiple daily inhalations and occasionally harsh intravenous antibiotic treatments are prescribed to most pwCF[3, 5]. Understanding and monitoring the mechanisms underlying the heterogeneous disease progression in CF is of critical clinical importance, as it could pave the way for personalized antibiotics administration[6].

To explain the heterogeneity in lung function deterioration in CF, previous studies have searched for possible immunological defects in pwCF. For example, a more pronounced Th2 immune response has been linked with deleterious outcomes in people with *Pseudomonas aeruginosa* infections, while a Th1-skewed immune response seems more protective[7, 8]. The direction of the adaptive immune response might be stimulated and influenced by cytokines released from innate pathways. Consistently, a reduced stimulated toll-like-receptor (TLR)-mediated cytokine response has been associated with a lower percent predicted forced expiratory volume in the first second (ppFEV₁)[9], as well as to a higher frequency of *P. aeruginosa* infections[10]. Furthermore, former studies have reported that IL-17A levels in the airways were positively correlated to clinical exacerbations[11], and unstimulated IL-8 levels in the blood were positively correlated to more severe disease[12]. Hence, we hypothesized that severe CF airway disease is associated with high unstimulated cytokine release causing reduced responsiveness of TLRs, which further leads to a disturbed or impaired immune response. In addition, baseline levels and changes in cytokine release during antibiotic treatment might be useful as biomarkers, which could indicate reversible and treatable flares in lung inflammation.

Studying and evaluating the CF immune system in clinical settings remains challenging, as non-standardized methods are the only available tools. This might explain why studies of the CF immune response have found varying and at times inconsistent tendencies[9, 12]. A new standardized immune assay (TruCulture - Myriad RBM, Austin, TX, US) has been developed and tested as a predictive and diagnostic tool to link systemic immune responses to clinical parameters in several clinical cohorts, including people undergoing surgery, people with cancer and people

with neuroborreliosis[13–16]. TruCulture estimates unstimulated and stimulated cytokine release to TLR agonists in whole blood using test tubes containing standardized concentrations of different agonists. As such, the *in vitro* stimulation of whole blood with these agonists mimics the *in vivo* immune response to external stimuli[16]. To our knowledge, TruCulture has never been used to study the immunological responses in CF.

This exploratory study evaluated the TruCulture assay in whole blood as a research and clinical tool in pwCF. In particular, we tested the ability of TruCulture to predict and explain differences in lung function, measured as ppFEV₁. Further, to investigate the use of TruCulture as a clinical tool, we assessed the immunomodulatory effects of systemic antibiotic treatment and the assay's ability to predict and explain the clinical efficacy of a 14-day course of intravenous antibiotic treatment in pwCF.

Methods

Study design

This single-centre study combined prospective and longitudinal data collected between 2019 and 2020 at the Copenhagen CF Centre, Rigshospitalet, Denmark. The cross-sectional arm enrolled patients attending routine consultations, and the longitudinal arm enrolled patients at the start of intravenous antibiotic treatments. In the Copenhagen CF Centre, pwCF with chronic *P. aeruginosa* infection are treated with quarterly elective 14-day treatments and with urgent treatments in case of exacerbations[17]. Blood samples were collected during routine consultations and at the start and end of intravenous antibiotic treatment. Data from the first visit of all participants were included in the cross-sectional analyses.

Participants

pwCF older than 10 years with chronic *P. aeruginosa* infections were eligible for the study. Chronic infection with *P. aeruginosa* was confirmed by routine microbiology culturing either from sputum samples or endo-laryngeal suction samples from the monthly clinical controls. Infection was defined as chronic when present in $\geq 50\%$ of at least three microbiological cultures within the last year[18]. However, due to limited sputum sampling during the COVID-19 pandemic, we allowed individuals, who had met these criteria in previous years, if whole genome sequencing and levels of *P. aeruginosa* antibodies supported the diagnosis.

To ensure the inclusion of participants with different levels of inflammation in the study samples, we enrolled pwCF of different ages, sex, and lung function as assessed by ppFEV₁. Furthermore,

we also included 6 adolescents without *P. aeruginosa* to enable the inclusion of participants with less lung inflammation (Table 1). Lung transplant recipients were excluded.

TruCulture assay

Blood samples were collected in lithium heparin tubes. Sixty minutes after sampling, 1 mL of whole blood was added to each TruCulture test tube. Our assay included three different TLR agonists: Polyinosinic:polycytidylic acid (Poly I:C, a TLR3 agonist), which mimics double stranded RNA often found in vira[19]; lipopolysaccharide[20] (LPS, a TLR4 agonist), which is an endotoxin from *Escherichia coli*, O111:B4; and Resiquimod 848[21] (R848, a TLR7/8 agonist), which mimics viral single stranded RNA[22]. A fourth tube contained only a test medium with no stimulating effects (NULL). The tubes were incubated for 22 hours at 37°C, after which release of TNF- α , IL-1 β , IL-6, IL-8, IL-10, IL-12p40, IL-17A, IFN- α and IFN- γ was measured in the supernatant by a multiplex Luminex 9-plex assay, as indicators of immune function.

Biochemistry

Additional blood samples to determine levels of haemoglobin (absorption photometry, Sysmex XN (Sysmex Co., Kobe, Japan), thrombocytes (particle count, resistance measurement, Sysmex XN) and leucocytes including differential count (particle count, flowcytometry, Sysmex XN) were analysed at the same day as the sample for TruCulture.

Lung function and baseline data

During clinical consultations, lung function was measured with spirometry (Intramedic Vyntus™ SPIRO, reference: Standard EU-GLI[23]). Baseline data included demographic data (age, sex), clinical data (ppFEV₁, CF mutation class, cystic fibrosis-related diabetes (CFRD) and pancreatic insufficiency status (y/n), and body mass index) and microbiological data (duration of *P. aeruginosa* infection and chronic infection with other CF-related pathogens) (Table 1).

Statistical analysis

Correlations between ppFEV₁ and monocytes, neutrophils and lymphocytes were tested with Spearman's correlation analyses. The cytokine profiles in the different TLR assays were illustrated in box plots including interquartile ranges (IQR: 25th-75th percentile) based on an internal reference from healthy individuals. With one-sample Wilcoxon signed-rank tests, we tested whether the cytokine release in the CF population was below or above the interquartile range of the reference population. Associations between log-transformed cytokine release and baseline (before antibiotic treatment) ppFEV₁ were evaluated in univariate and multivariate models adjusted for neutrophils, monocytes, lymphocytes and the TruCulture batch, as the batches were

changed with 12 months intervals, due to expiry. In some cases, the cytokine release was below the detection threshold, and therefore we used Tobit regression models, which accounts for left censoring of the data by adding detection threshold values to the model. Changes in the cytokine release after antibiotic treatment was presented as the pseudo-median of the symmetric percentage change and tested with one-sample Wilcoxon signed-rank test. Symmetric percentage changes in cytokine profiles, ppFEV₁, and differential counts after a 14-day intravenous antibiotic course were correlated with Spearman's correlation and presented in heat maps. A p-value <0.05 was considered significant. In supplementary analyses, we used principal component analyses to investigate the relation between cytokine release profiles and age, lung function, sex or modulator treatment. Moreover, in boxplots we showed differences in cytokine release in pwCF with and without *P. aeruginosa* infections.

Ethics

The study was approved by the Ethical Committee of the Capital Region of Denmark (H-19001151), and by the local data protection agency in the Capital Region of Denmark (P-2020-1191). Oral and written consent were given before sample collection by the participants.

Results

The baseline characteristics of the 52 participants in the cross-sectional (all) and longitudinal study (n=24, 46% of total) are presented in Table 1. The median age was 36 years (IQR: 19; 46), and based on ppFEV₁ at baseline, 23 (44%) had mild (ppFEV₁>70), 22 (42%) had moderate (ppFEV₁ 40-70) and 7 (13%) had severe lung disease (ppFEV₁<40). The genotype was classified as severe in 90% of participants (class I-III), and 14 (27%) of the participants had chronic lung infections with pathogens other than *P. aeruginosa*, e.g., *Burkholderia* spp. and *Achromobacter* spp. In the longitudinal study group, the median ppFEV₁ at follow-up was lower than at baseline. Most patients (79%) were on CFTR modulator therapy, but only 10% had started elexacaftor/tezacaftor/ivacaftor (ETI) at enrolment. At baseline, ppFEV₁ correlated negatively with the count of neutrophils (rho = -0.3, p=0.03) but did not correlate with lymphocytes (rho = 0.2, p = 0.15) and monocytes (rho = -0.15, p = 0.29). There was no apparent variation in the cytokine release profiles depending on age, lung function, sex and modulator treatment in principal component analyses (Supplementary Figure 1).

Cytokine release in pwCF compared to healthy people – the cross-sectional study

Figure 1 shows the unstimulated and stimulated cytokine release in the CF population compared to reference values. While the unstimulated cytokine release did not differ between pwCF and

healthy individuals, a high proportion of TLR-stimulated cytokine release was lower in pwCF. When stimulating TLR3, TLR4, and TLR7/8, the release of IFN- γ ($p < 0.001$, $p < 0.001$, and $p < 0.001$), and IL-12p40 ($p = 0.007$, $p = 0.045$, and $p < 0.001$) were below the lower quartile range of the healthy individuals. Similarly, IFN- α release was reduced after TLR4 and TLR7/8 stimulation ($p < 0.001$ and $p < 0.001$) and IL-1 β release was reduced after TLR3 and TLR4 stimulation ($p = 0.009$ and $p < 0.001$). IL-6 ($p < 0.001$) and TNF- α ($p < 0.001$) release were also reduced in the TLR3 stimulated samples and IL-10 ($p < 0.001$) release was reduced in the TLR7/8 stimulated samples. In a sensitivity analysis, pwCF without *P. aeruginosa* seemed to have increased TLR3-stimulated cytokine release compared to pwCF with chronic *P. aeruginosa* infection (Supplementary Figure 2).

The relation between cytokine release and lung function in pwCF – the cross-sectional study
The correlations between cytokine release and ppFEV₁ are shown in Table 2. In the unadjusted models, positive associations were found between ppFEV₁ and TLR3-stimulated release of IL-1 β , IL-6, and TNF- α . The associations with TLR3-stimulated IL-1 β and IL-6 remained significant in the adjusted model ($p = 0.02$ and $p = 0.01$, respectively). Furthermore, in the adjusted model, ppFEV₁ was positively associated with TLR4-stimulated IL-6 and negatively associated with unstimulated IL-17A.

Changes in cytokine release after antibiotic treatment in pwCF – the longitudinal study
Figure 2 shows the changes in TLR-stimulated cytokine release after 14 days of intravenous antibiotic treatment. The most significant change after treatment was found in the TLR3-stimulated and unstimulated samples. The TLR3-stimulated cytokine release tended to be stronger after treatment, and IL-17A and IL-1 β increased significantly ($p = 0.023$ and $p = 0.046$, respectively). In contrast, the unstimulated samples tended to have lower cytokine release after 14 days of antibiotics, and IFN- γ and IL-6 declined significantly ($p = 0.011$ and $p = 0.044$, respectively).

The relation between cytokine release and the change in lung function in pwCF - the longitudinal study

The correlations between baseline cytokine release and the corresponding changes in lung function and differential leukocyte counts after a 14-day intravenous antibiotic course are shown in Figure 3A. Except for a negative correlation between the baseline TLR4-stimulated IFN- γ release and unstimulated IFN- α and the change in ppFEV₁, no associations were identified between baseline cytokine release and lung function. Figure 3B shows correlations between changes in all measures: cytokine release, lung function, and differential leukocyte counts. Increasing release of TLR7/8-stimulated IL-8 correlated with decreased lung function, whereas increased release of

TLR3-stimulated IFN- α and IL-6 and unstimulated IFN- α were associated with increased ppFEV₁. In general, changes in the TLR7/8 stimulated cytokines seemed to correlate with changes in leukocyte and neutrophil counts observed after 14 days of intravenous antibiotics.

Discussion

We evaluated lung function and immune response in 52 pwCF using TruCulture assay. Herein, we found that pwCF had normal release of cytokines in the unstimulated samples. However, a lower level of cytokines compared to a healthy population could be detected upon stimulation, and this was especially clear for Th1-related cytokines such as IFN- γ , IL-12p40, and IL-1 β . In the cross-sectional study, TLR3-stimulated IL-1 β and IL-6 were positively associated with ppFEV₁ in all statistical models. In the longitudinal study, TLR3-stimulated IL-17A and IL-1 β increased by approximately 37% and 45% over the course of intravenous antibiotic treatment, and unstimulated IFN- γ and IL-6 decreased by 93% and 66%, respectively. However, cytokine release at the baseline visit did not seem to be associated with changes in lung function during the antibiotic treatment.

Cross-sectional study

In the cross-sectional part of our study, we used TruCulture as an explorative tool to look for immunological deviations that might explain inter-individual differences in the progression of CF lung disease. Comparing cytokine release from pwCF to samples from a healthy population, we found that the unstimulated samples were similar in pwCF and healthy controls, but a high proportion of the stimulated cytokines were significantly lower in pwCF than in healthy controls. This was particularly clear for IFN- γ in all TLR-stimulated assays, where more than 75% of the CF population had cytokine release below the lower quartile range of healthy controls. Similarly, IL-12p40 was significantly lower in all stimulated assays, and in the TLR3-stimulated assay IL-1 β , IL-6, and TNF- α were also reduced. Furthermore, a consistent pattern was found when comparing pwCF with and without chronic *P. aeruginosa*, as pwCF with chronic infection seemed to have a lower stimulated cytokine release. However, the sample size was too small to draw firm conclusions. It has previously been described that high *ex vivo* unstimulated cytokine release and low stimulated cytokine release may reflect exhaustion[14]. Thus, our data indicate that the immune system in CF might be exhausted or impaired after long-term persistent infection and inflammation[24], similar to what is seen in other types of inflammatory diseases[25]. This could be explained by chronic infections or tissue injury, which negatively regulates the TLR-stimulated immune response[26]. Though the new highly effective modulator therapy (ETI) might mitigate

some of this exhaustion by e.g., improving inflammation, this was beyond the scope of this paper to investigate. Nevertheless, exhaustion was evident in this cohort, where the majority were treated with the first-generation modulators.

Immune exhaustion assessed with TruCulture has also been found in people infected with *Borrelia burgdorferi*[15]. In this study, similar indicators of immune exhaustion with generally reduced cytokine release profiles were found in the *Borrelia* group compared to the healthy group. Though CF and neuroborreliosis have very different manifestations, both diseases may be characterized by long-term, low-grade inflammation, possibly explaining their low TLR response.

In previous studies, it has been suggested that immune exhaustion may be linked with clinical status in CF: high *unstimulated* release of IL-8 was associated with more severe disease[12], and at the same time, low release of *TLR-stimulated* IL-8 was associated with low ppFEV₁[9]. In addition, Brazova et al. showed that low release of LPS-stimulated IFN- γ and IL-4 in children with CF predicted impaired lung function at 3-year follow-up visits[10], thus supporting a link between immunological exhaustion and clinical parameters in CF. In our cross-sectional study, a few findings were compatible with these observations. For instance, unstimulated IL-17A was negatively associated with ppFEV₁ in the adjusted model, whereas TLR3-stimulated IL-1 β and IL-6 were positively associated with ppFEV₁ in both statistical models (Table 2). In summary, our cross-sectional study found a pattern of an exhausted immune system and signs that this may be associated with lower ppFEV₁. This may imply that a weak TLR-dependent response indicates inflammation and poor clinical status in CF, as hypothesized. However, while some of our findings may support our initial hypothesis, there was not a clear association between cytokine release and ppFEV₁, in contrast to previous studies[9, 12].

Longitudinal study

In the longitudinal part of the study, we tested TruCulture as a clinical tool to predict and explain the effect of a 14-day treatment with intravenous antibiotics, reflected by the ppFEV₁. Figure 2 shows the changes in cytokine release after 14 days, where the unstimulated release of IFN- γ and IL-6 were significantly decreased after antibiotic treatment. The TLR3-stimulated cytokine release was also noticeable in the longitudinal study, with cytokine release tending to be enhanced after antibiotic treatment and significantly increased for IL-17A and IL-1 β (Figure 2). With a tendency of increased stimulated cytokine release and lower unstimulated release, these changes may reflect a recovering immune system after antibiotic treatment. Unfortunately, the changes did not appear

to correlate with changes in lung function after 14 days (Figure 3). However, we acknowledge that the follow-up time may have been too short. In Figure 3A, we observed that a few cytokine releases (low baseline release of TLR4-stimulated IFN- γ and unstimulated IFN- α) were associated with a treatment related increase in ppFEV₁. However, based on our findings we question whether TruCulture would be useful as a clinical tool to predict the effect of intravenous treatment in pwCF. In Figure 3B, a treatment-related increase in TLR3-stimulated IFN- α and IL-6 were associated with an increase in ppFEV₁, which seems in line with our initial theory. On the other hand, and in contrast to our expectations, a decrease in TLR7/8 stimulated IL-8 and an increase in unstimulated IFN- α were associated with higher ppFEV₁ after 14 days of intravenous antibiotics. We speculate whether these unexpected correlations and the decline in ppFEV₁ after treatment may represent a treatment-related immunological flare due to release of increased cell debris.

Another interesting finding of our study was the apparent significance of TLR3-stimulated cytokines, which were related to both lung function levels (Table 2) and affected by antibiotic treatment (Figure 2). TLR3 is usually activated by dsRNA, but also seems to respond to other heterogenous RNA species found in necrotic cell debris[27]. This may suggest that high amounts of cellular debris in severely injured lungs or other organs cause downregulation of the TLR3-stimulated cytokine release in pwCF. However, the causality cannot be confirmed in this study, and another explanation could therefore be that low TLR3-stimulated cytokine release leads to worse lung disease in CF. Pursuing TLR3-stimulated cytokines in future studies may be highly interesting, since other studies have favoured using the TLR4 agonist LPS when examining the stimulated innate immune response in the CF lungs[10, 12].

Strengths and limitations

An important strength of this study is that it is one of the few studies that thoroughly explore the innate immune system in CF. Moreover, we included pwCF receiving elective antibiotic treatments. This suggests that our study population was clinically stable, with few outliers due to acute exacerbations. However, our study also has some limitations. First, it is essential to note that ppFEV₁ did not increase in our study population after antibiotic treatment. Thus, our population did not respond as expected to the 14-day intravenous treatment, suggesting that the study may have been underpowered. The effect may have been further attenuated by a high mean age in our study population, where lung function may be less influenced by elective antibiotic treatment due to long term chronic inflammation. In our regression analyses comparing baseline lung function and cytokine release, only few associations had similar estimates in the adjusted and unadjusted

model. There is currently no gold standard for confounder adjustment in TruCulture. Hence, the associations which were only found after adjusting should be interpreted with caution, while associations persisting in both models may be considered more robust and reliable. Furthermore, TruCulture is based on circulating blood, why we might have overlooked some aberrant patterns in the CF immune system, as the CF inflammation is often less systemic and more localized in the lungs. This could also explain the scarce signals without a systematic pattern in this study. However, another possible explanation may simply lie in the choice of stimuli and cytokines assessed, thus a different combination of TLR-stimuli and cytokines within the TruCulture framework could potentially have revealed important immunological patterns in CF. To improve interpretability of future findings, future studies may benefit from combined assessments of systemic and local cytokine release, repeated measurements, longer follow-up time, and larger assay panels of innate immunological pathways.

Conclusion

In this first study of TruCulture immune response in pwCF and chronic infection with *P. aeruginosa*, we found that the TLR-stimulated cytokine release appeared lower in pwCF compared to healthy individuals. This likely reflects immune exhaustion, i.e., an acquired or inherent immune deficiency in CF. Antibiotic treatment seemed to affect the pattern of cytokine release, which may reflect recovery of the immune response. However, the cytokine release did neither convincingly predict ppFEV₁ nor the change in ppFEV₁ after antibiotic treatment in our CF population and we did not identify any important immunological cause of variation in CF lung disease progression. This could have been due to systemic, rather than local, examination of an incorrect selection of cytokines. Thus, based on our initial findings, TruCulture may not be an optimal clinical tool to predict changes in lung function in pwCF. However, the TLR3-stimulated cytokine release was identified as a potential marker of clinical variations in CF, though no causal pathways were identified. This suggests further research in the TLR3 response in CF.

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Table 1: Baseline data of 52 people with cystic fibrosis

	All participants n = 52	Only cross-sectional study n = 28	Longitudinal study n = 24
Demographics			
Age (years): median (IQR)	36 (19, 46)	36 (20, 44)	34 (18, 47)
Female: n (%)	22 (42%)	12 (43%)	10 (42%)
Mutation			
Severe mutation (class I-III): n (%)	47 (90%)	23 (82%)	24 (100%)
Mild mutation (class IV-V): n (%)	5 (10%)	5 (18%)	0 (0%)
Lung disease			
Baseline ppFEV ₁ :			
Median (IQR)	65 (50, 81)	66 (58, 79)	62 (41, 83)
Mild lung disease (ppFEV ₁ >70): n (%)	23 (44%)	13 (46%)	10 (42%)
Moderate lung disease (ppFEV ₁ 40-70): n (%)	22 (42%)	12 (43%)	10 (42%)
Severe lung disease (ppFEV ₁ <40): n (%)	7 (13%)	3 (11%)	4 (17%)
Follow-up ppFEV ₁ : median (IQR)	54 (45, 84)		54 (45, 84)
Chronic <i>P. aeruginosa</i> with no chronic co-infection*: n (%)	35 (67%)	18 (64%)	17 (71%)
Chronic <i>P. aeruginosa</i> with chronic co-infection*: n (%)	11 (21%)	4 (14%)	7 (29%)
Time since first <i>P. aeruginosa</i> (years): median (IQR)	26 (7, 39)	24 (7, 35)	27 (9, 41)
Chronic infection with other pathogens*, no <i>P. aeruginosa</i> : n (%)	3 (6%)	3 (11%)	0 (0%)
Free of CF related chronic infection: n (%)	3 (6%)	3 (11%)	0 (0%)
Leukocytes in plasma: median (IQR)	8 (6, 10)	7 (6, 10)	8 (6, 10)
Metabolism			
Cystic fibrosis-related diabetes: n (%)	15 (29%)	8 (29%)	7 (29%)
Pancreatic insufficiency: n (%)	50 (96%)	26 (93%)	24 (100%)
Body mass index: mean (sd)	21 (3)	21 (3)	21 (3)
Treatment			
Any modulator: n (%)	41 (79%)	23 (82%)	18 (75%)
Tezacaftor/ivacaftor: n (%)	25 (48%)	14 (50%)	11 (46%)
Ivacaftor/lumacaftor: n (%)	9 (17%)	6 (21%)	3 (12%)
Ivacaftor: n (%)	2 (4%)	2 (7%)	0 (0%)
Elexacaftor/tezacaftor/ivacaftor: n (%)	5 (10%)	1 (4%)	4 (17%)
Azithromycin: n (%)	34 (65%)	16 (57%)	18 (75%)

Data is shown as count (percentage), mean (standard deviation) and median (interquartile range) as appropriate. One person in the cross-sectional study had no differential leukocyte count and four participants in the longitudinal study had no spirometry at 14-day follow-up. Data on duration of *Pseudomonas aeruginosa* was missing for four persons in the longitudinal study.

*Pathogens considered as cause of chronic co-infection included: *Achromobacter* spp., *Burkholderia* spp., Nontuberculous mycobacterial infections including *Mycobacterium abscessus* and *Mycobacterium avium* complex, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Escherichia coli*, *Proteus mirabilis* and *Aspergillus* spp. Abbreviations: IQR; interquartile range, sd; standard deviation, ppFEV₁; percent of predicted forced expiratory volume in first second, *P. aeruginosa*; *Pseudomonas aeruginosa*, CFTR; Cystic fibrosis transmembrane conductance regulator, Triple modulator; elexacaftor/tezacaftor/ivacaftor, spp.; species.

Table 2: Correlations between cytokine release and ppFEV₁ in 52 people with cystic fibrosis

Stimuli	Cytokine	Unadjusted model				Adjusted model			
		e ^β	95 % CI lower	95 % CI upper	p-value	e ^β	95 % CI lower	95 % CI upper	p-value
Poly I:C (TLR3)	IFN-α	1.02	1.00	1.04	0.11	1.01	0.99	1.04	0.26
	IFN-γ	1.02	0.99	1.04	0.14	1.01	0.98	1.03	0.71
	IL-10	1.00	0.99	1.01	0.85	1.00	0.99	1.01	0.69
	IL-12p40	1.00	0.97	1.03	0.93	0.99	0.96	1.02	0.59
	IL-17A	1.01	0.99	1.02	0.34	1.00	0.99	1.01	0.81
	IL-1β	1.02	1.01	1.04	0.004*	1.02	1.00	1.04	0.02*
	IL-6	1.03	1.01	1.04	<0.001*	1.02	1.01	1.04	0.01*
	IL-8	1.00	0.99	1.01	0.83	0.99	0.99	1.00	0.19
	TNF-α	1.02	1.01	1.04	0.003*	1.01	1.00	1.03	0.11
LPS (TLR4)	IFN-α	1.00	0.99	1.01	0.90	1.00	0.99	1.00	0.50
	IFN-γ	1.01	1.00	1.02	0.12	1.01	0.99	1.02	0.45
	IL-10	1.00	0.99	1.01	0.92	1.00	0.99	1.01	0.73
	IL-12p40	1.00	1.00	1.01	0.25	1.00	1.00	1.01	0.34
	IL-17A	1.00	0.99	1.01	0.78	1.00	0.99	1.01	0.97
	IL-1β	1.00	0.99	1.01	0.52	1.01	1.00	1.01	0.27
	IL-6	1.01	1.00	1.01	0.07	1.01	1.00	1.01	0.01*
	IL-8	1.00	1.00	1.00	0.80	1.00	1.00	1.00	0.98
	TNF-α	1.00	0.99	1.02	0.90	0.99	0.98	1.01	0.45
R848 (TLR7/8)	IFN-α	1.01	1.00	1.03	0.06	1.00	0.99	1.02	0.45
	IFN-γ	1.01	0.99	1.02	0.38	1.00	0.98	1.02	0.88
	IL-10	1.00	0.99	1.01	0.78	1.00	0.99	1.01	0.72
	IL-12p40	1.00	0.99	1.01	0.40	1.00	0.99	1.01	0.78
	IL-17A	1.00	0.99	1.01	0.83	1.00	0.99	1.01	0.71
	IL-1β	1.00	0.99	1.01	0.93	1.00	0.99	1.01	0.73
	IL-6	1.00	1.00	1.01	0.22	1.01	1.00	1.01	0.14
	IL-8	0.99	0.99	1.00	0.11	0.99	0.99	1.00	0.07
	TNF-α	1.00	0.98	1.02	0.97	0.99	0.97	1.01	0.20
NULL	IFN-α	1.00	0.99	1.02	0.66	1.00	0.99	1.01	0.84
	IFN-γ	0.99	0.97	1.01	0.52	1.00	0.98	1.03	0.96
	IL-10	1.00	0.99	1.01	0.91	1.00	0.99	1.01	0.89
	IL-12p40	0.99	0.95	1.04	0.66	0.98	0.93	1.04	0.53
	IL-17A	1.00	0.98	1.01	0.72	0.99	0.97	1.00	0.02*
	IL-1β	1.00	0.98	1.02	0.96	1.00	0.98	1.02	0.89
	IL-6	0.99	0.98	1.01	0.41	1.00	0.98	1.01	0.73
	IL-8	0.99	0.98	1.01	0.37	0.99	0.97	1.00	0.06
	TNF-α	0.99	0.98	1.01	0.37	1.00	0.99	1.01	0.73

Cytokine release was modelled as the outcome and ppFEV₁ as the independent variable in Tobit regressions. Cytokine release was log-transformed prior to the analysis and coefficients from the regressions were presented as back-transformed estimates. The multivariate model is adjusted for neutrophils, monocytes and lymphocytes and the TruCulture batch.

*Significant with a 95% confidence level. Abbreviations: ppFEV₁; percent of predicted forced expiratory volume in first second, CI; confidence interval, IFN; interferon, IL; interleukin, TNF; tumor necrosis factor, TLR, Toll-like receptor, Poly I:C; Polyinosinic:polycytidylic acid, LPS; lipopolysaccharide, R848; Resiquimod 848.

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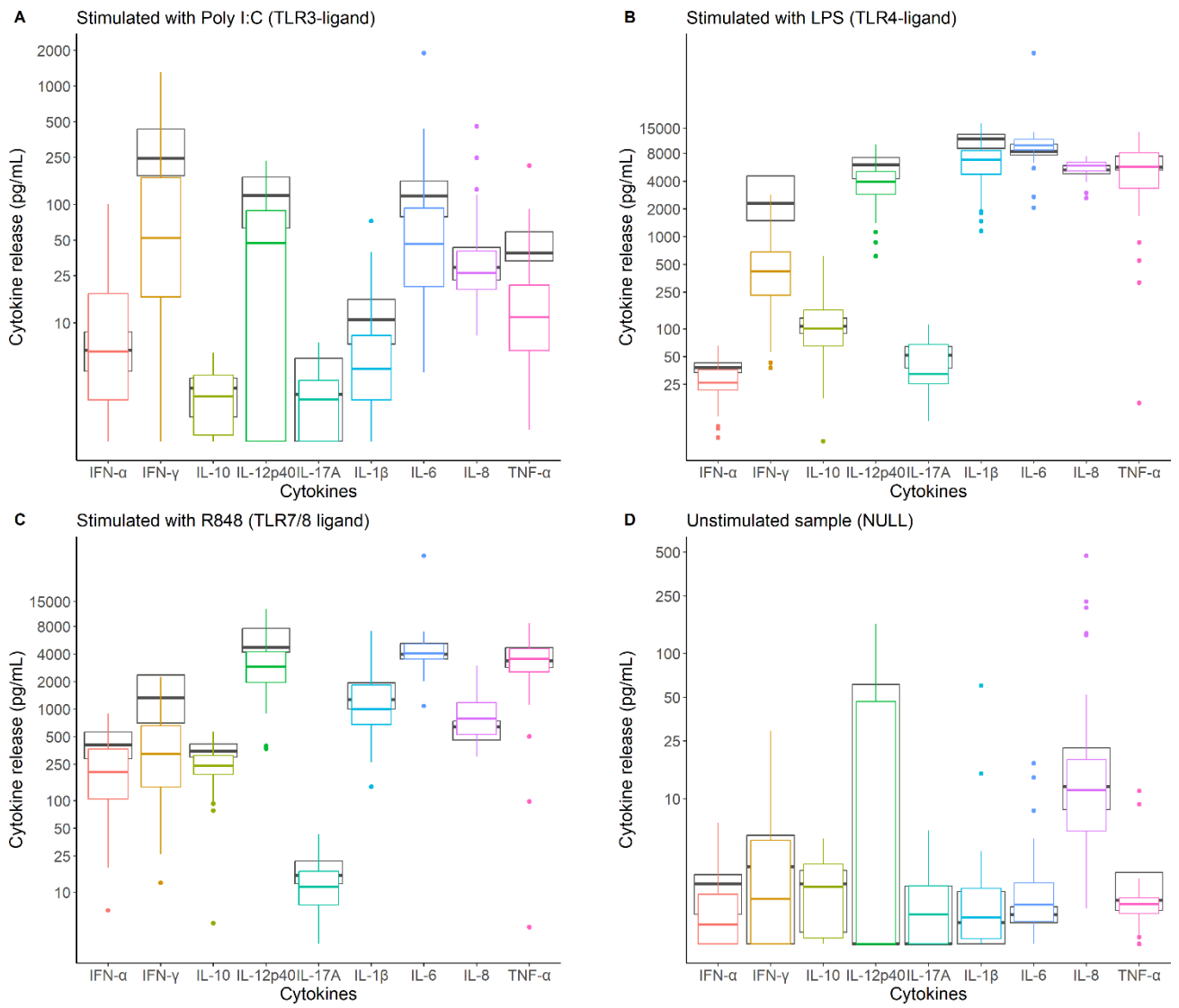


Figure 1

Symmetric percent change (%)

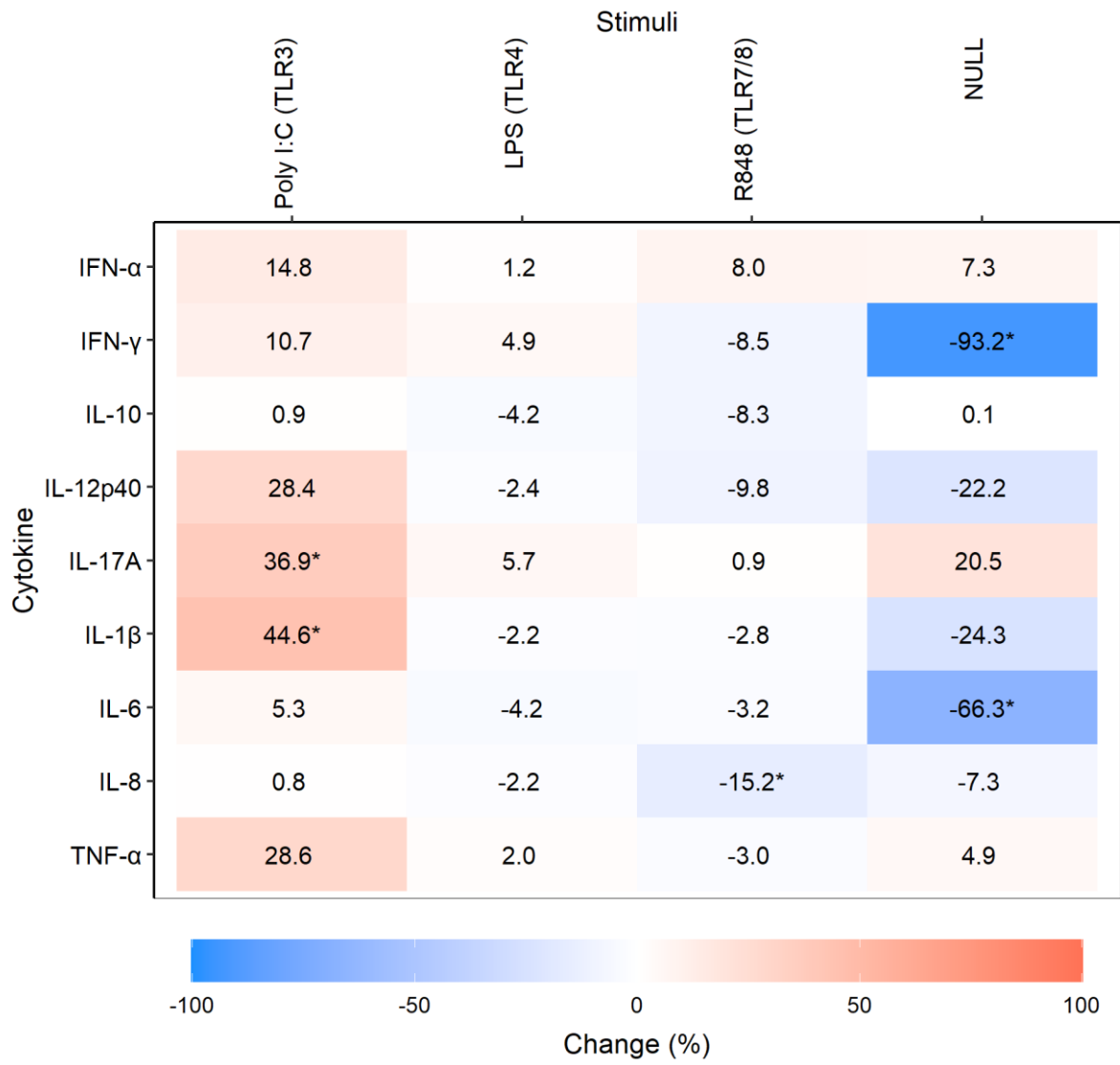


Figure 2

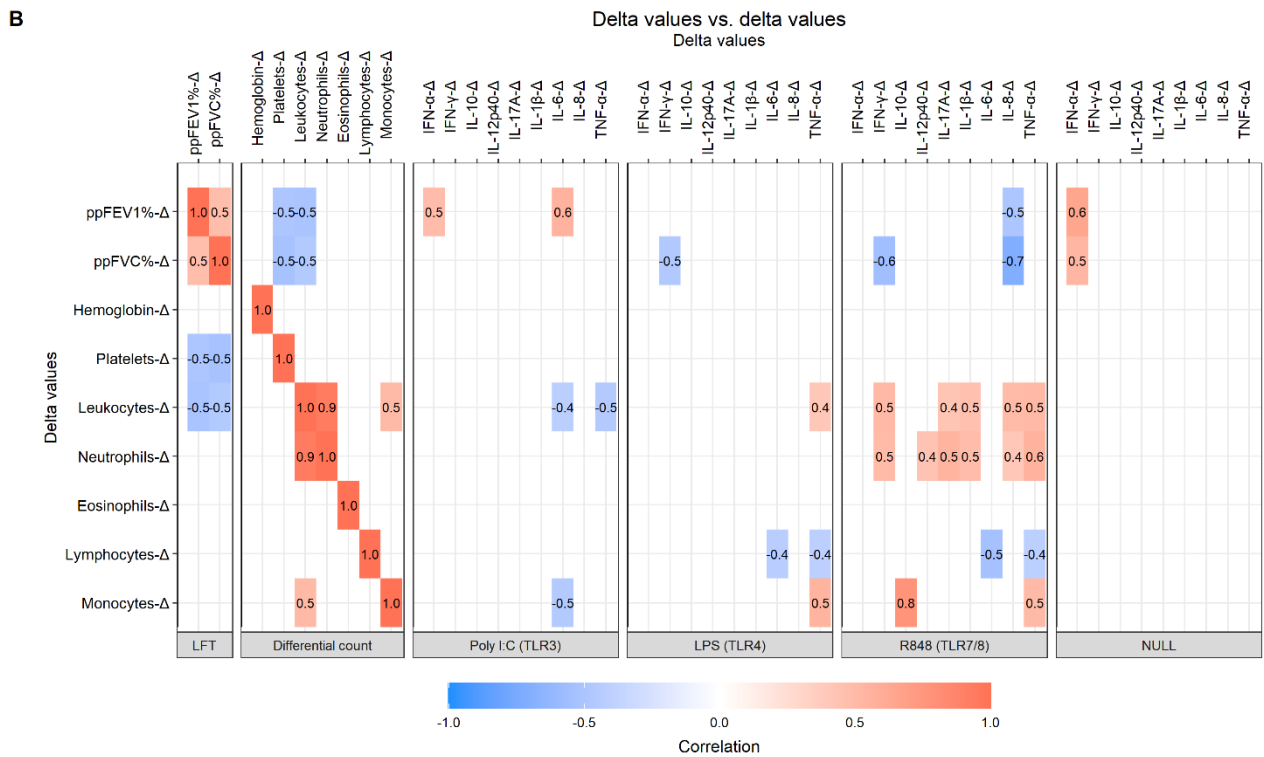
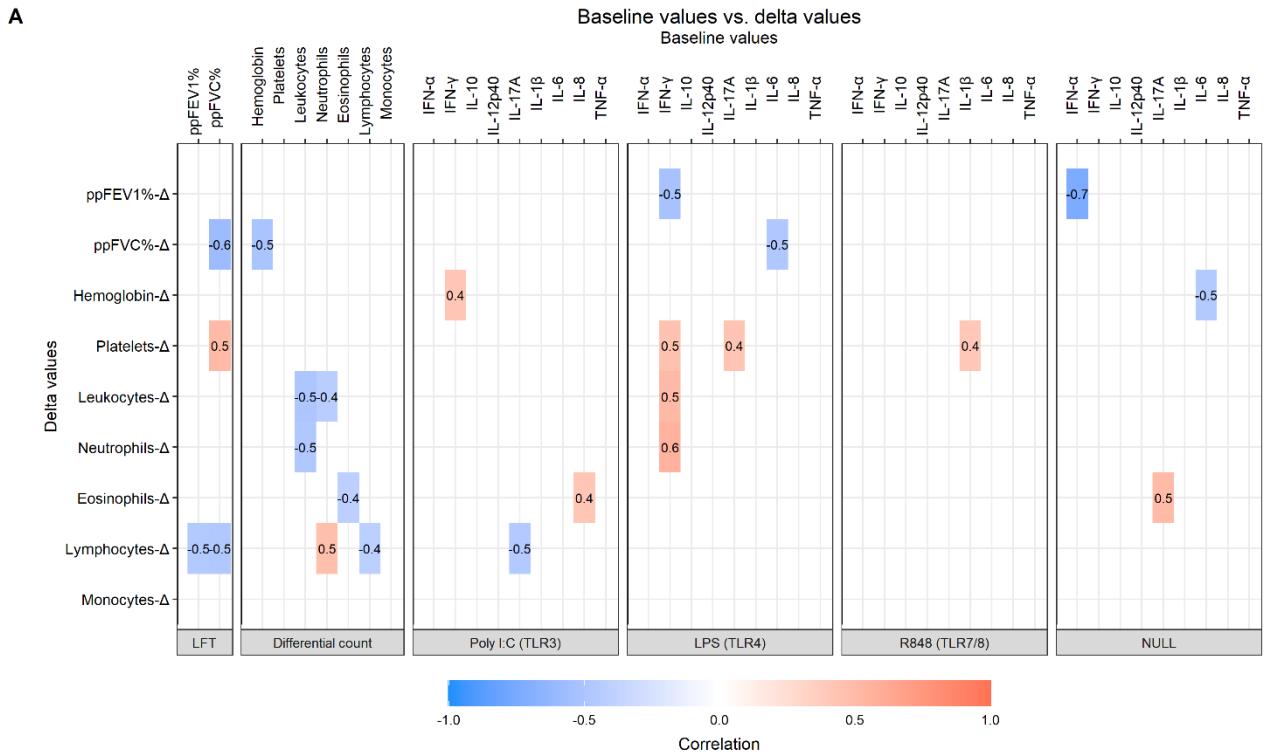
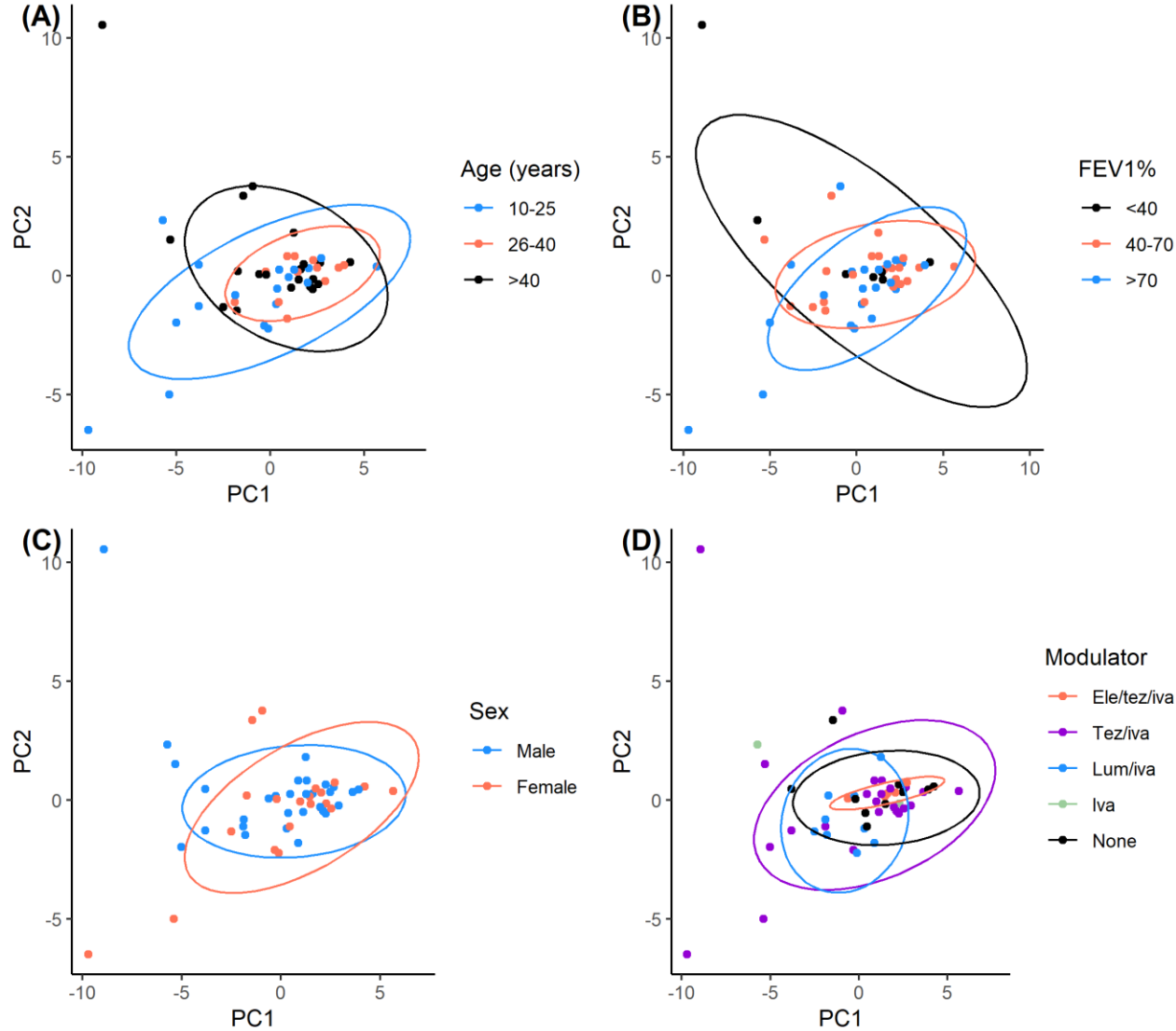


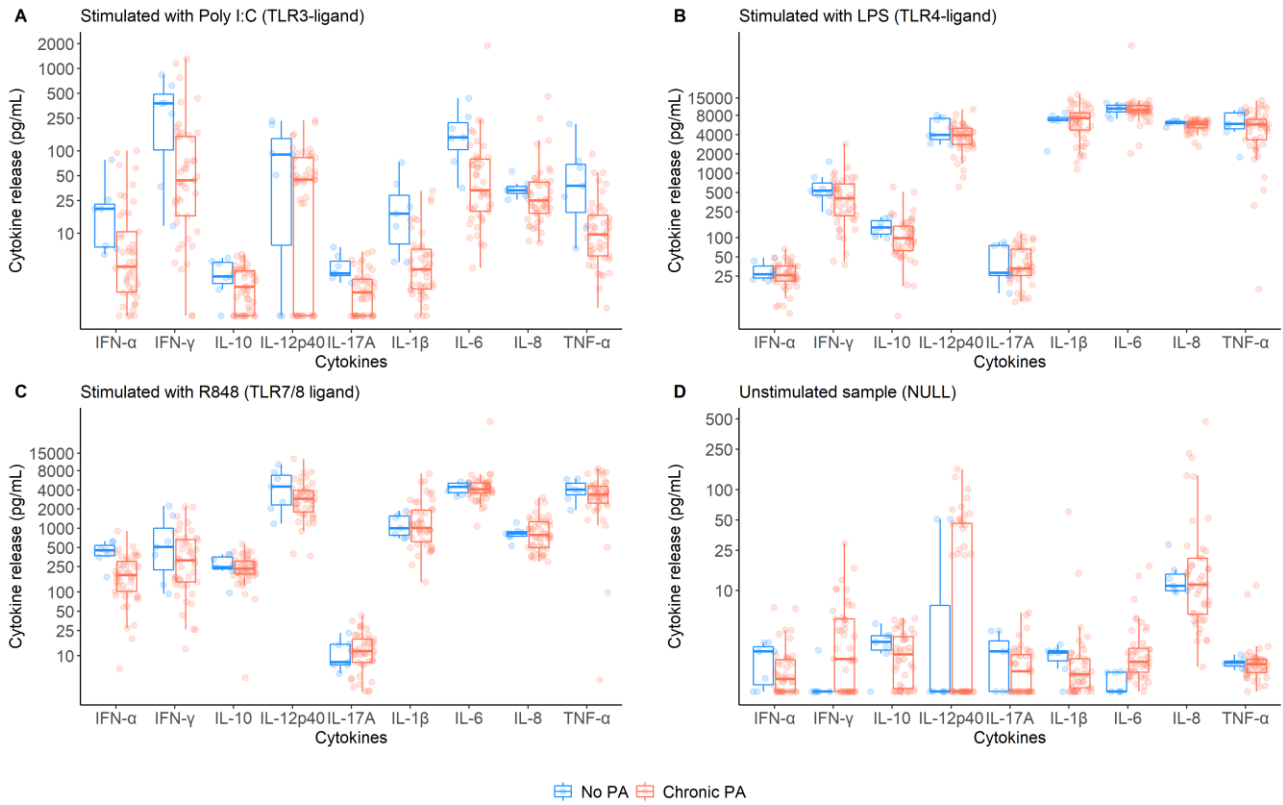
Figure 3

Supplementary Figure 1: Principal component analysis of stimulated cytokine release in people with cystic fibrosis



Supplementary Figure 1: Cytokine release summarized with two principal components from people with CF. Abbreviations: PC; principal component, ele; elexacaftor, tez; tezacaftor, iva; ivacaftor.

Supplementary Figure 2: Cytokine release in 52 people with cystic fibrosis with and without *Pseudomonas aeruginosa* lung infection



Supplementary Figure 2: The figure shows cytokine release in response to different stimuli in TruCulture test tubes from 52 people with CF, comparing 6 people with no chronic *Pseudomonas aeruginosa* (blue) and 46 people with chronic *Pseudomonas aeruginosa* infection (red). Coloured boxes represent 25th percentiles, medians, and 75th percentiles, the coloured points represent individual measurements. Cytokine release is shown in picograms per millilitre on a logarithmic scale. Abbreviations: IFN; interferon, IL; interleukin, TNF; tumor necrosis factor.