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Original Article

Macrophage PD-1 associates with neutrophilia and reduced bacterial killing in early cystic fibrosis airway disease



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ABSTRACT

Background: Macrophages are the major resident immune cells in human airways coordinating responses to infection and injury. In cystic fibrosis (CF), neutrophils are recruited to the airways shortly after birth, and actively exocytose damaging enzymes prior to chronic infection, suggesting a potential defect in macrophage immunomodulatory function. Signaling through the exhaustion marker programmed death protein 1 (PD-1) controls macrophage function in cancer, sepsis, and airway infection. Therefore, we sought to identify potential associations between macrophage PD-1 and markers of airway disease in children with CF.

Methods: Blood and bronchoalveolar lavage fluid (BALF) were collected from 45 children with CF aged 3 to 62 months and structural lung damage was quantified by computed tomography. The phenotype of airway leukocytes was assessed by flow cytometry, while the release of enzymes and immunomodulatory mediators by molecular assays.

Results: Airway macrophage PD-1 expression correlated positively with structural lung damage, neutrophilic inflammation, and infection. Interestingly, even in the absence of detectable infection, macrophage PD-1 expression was elevated and correlated with neutrophilic inflammation. In an *in vitro* model mimicking leukocyte recruitment into CF airways, soluble mediators derived from recruited neutrophils directly induced PD-1 expression on recruited monocytes/macrophages, suggesting a causal link

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between neutrophilic inflammation and macrophage PD-1 expression in CF. Finally, blockade of PD-1 in a short-term culture of CF BALF leukocytes resulted in improved pathogen clearance.

Conclusion: Taken together, these findings suggest that in early CF lung disease, PD-1 upregulation associates with airway macrophage exhaustion, neutrophil takeover, infection, and structural damage.

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1. Introduction

Resident macrophages play a key role in tissue homeostasis and responses to infection and injury [1]. In the airways, macrophages quickly adapt to variations in oxygen pressure, inflammation, and the presence of pathogens [2]. Exposure of airway macrophages at birth and thereafter to host- and microbe-derived factors shapes long-term responses and the likelihood to mount an aberrant response to later insults [3]. In cystic fibrosis (CF), airway epithelial cells and macrophages display intrinsic defects due to mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. In particular, mucin hyperconcentration and dehydration can alter airway macrophages [4], which may contribute to abnormal immune regulation, and subsequent recruitment and activation of neutrophils, with ensuing lung damage [5–7]. In itself, CFTR dysfunction in CF airway macrophages may result in impaired phagocytosis and pathogen clearance [8–10]. While the impact of intrinsic CFTR-linked defects on CF airway macrophage responses has been well studied, little is known about their regulation by extrinsic immunomodulatory pathways.

Programmed cell death protein-1 (PD-1) is an immune checkpoint protein that regulates cell behavior during immune responses, with best known functions related to T-cell control in cancer and viral infection [11]. Comparatively, the role of PD-1 in macrophages is less well described, however there is increasing evidence of its role in tumor-infiltrating myeloid cells [12]. In blood monocytes during sepsis and in tumor-associated macrophages, PD-1 expression is associated with reduced uptake of bacteria and cell debris, respectively, suggesting a role for this pathway in shaping macrophage function [13–15]. Recently, PD-1 signaling was also found to mediate extrinsic control of resident macrophages by immunosuppressive leukocytes recruited in a model of airway infection [16]. We previously showed that neutrophils recruited to CF airways undergo reprogramming, leading to complex functional changes [17–21]. Among those is the acquisition of immunosuppressive functions [22], including expression of the PD-1 ligand, PD-L1, observed in adult patients with CF [23].

The early and massive recruitment of neutrophils to the CF airways [20] suggests a change in the poise of resident airway macrophages, which have an essential role in repairing tissue damage and preventing untimely neutrophil recruitment to sites of injury [24]. Inhibitory signaling in airway macrophages may reduce their capacity for blocking neutrophil recruitment in the early stages of inflammation in CF airways [25], while simultaneously inhibiting their ability to clear bacteria. Since PD-1 signaling has been shown to downregulate macrophage poise in multiple inflammatory diseases [13,15,16], we hypothesized that expression of PD-1 on airway macrophages in young children with CF would associate with progressive airway disease, and that blocking this signaling cascade could enhance bacteria killing. Therefore, we investigated the presence of the PD-1 pathway in airway macrophages and its relationship with intrinsic and extrinsic inflammatory factors critical for the early stages of CF lung disease.

2. Results

2.1. CF airway macrophages express high levels of surface PD-1

To determine the presence of the PD-1 signaling pathway in early CF airway inflammation, immune cells from blood and bronchoalveolar lavage fluid (BALF) from 45 children with CF, aged 3 to 62 months, were analyzed by flow cytometry (Fig. S1). The cohort included equal numbers of male and female subjects, with a mean age of 30 months, and 11/45 subjects were diagnosed with proinflammatory pathogen infections. Twenty subjects were homozygous for F508del, twenty had compound heterozygous mutations with F508del, and five had other mutations (Table S1). These subjects were primarily White with pancreatic insufficiency, and none were prescribed CFTR modulators at the time of sample collection. For Perth cohort subjects, demographic data on genotype, sex and birth date were obtained from clinical records. Data on ethnicity, pancreatic sufficiency status and current treatment were recorded by parent questionnaires, with “data unavailable” indicating this was not answered on the questionnaire (Table S2).

PD-1 expression on airway macrophages was higher than on airway neutrophils and T cells, by 10- and 17-fold respectively (Fig. 1A). Clustering analysis of CF airway macrophages with the spanning tree progression analysis of density normalized events (SPADE) algorithm [26] revealed a distinct pattern of PD-1 expression in different airway macrophage clusters, highlighting the intrinsic diversity of this immune cell population with regards to the PD-1 pathway (Fig. 1B). Airway macrophages and T cells showed significant expression of the PD-1 ligands, PD-L1 and PD-L2 (Fig. 1C and 1D), suggesting potential for activation of PD-1 signaling. While we observed differences in expression of PD-L1 and PD-L2 between neutrophils, macrophages and T cells, these may be partially caused by outliers. Indeed, there were 5–6 outlier points for expression of PD-L1 and PD-L2 on macrophages and T cells, and there were four subjects common to these groups of outliers. However, these subjects did not differ from the rest of the cohort based on demographic or inflammatory outcomes.

Given the pattern of expression of PD-1 and its ligands on airway immune cells, we investigated their relationship with clinical variables of early CF lung disease. No significant relationships were observed between the clinical outcomes and the levels of expression of PD-L1 or PD-L2. However, in a subset of 24 children with CF, for whom flow cytometry data and date-matched CT scans were acquired, PD-1 expression on airway macrophages correlated with both the total score of structural lung damage (%Dis), ranging between 0 and 5% (Rho = 0.51, $p = 0.01$, Fig. 2A), and the score for bronchiectasis (%Bx), ranging from 0 and 2.1% (Rho = 0.47, $p = 0.01$, Fig. 2B). Furthermore, while PD-1 expression on airway macrophages did not correlate with absolute macrophage count (Rho = -0.03, $p = \text{n.s.}$, Fig. 2C), it correlated negatively with airway macrophage percentage (Rho = -0.57, $p = 0.003$, Fig. 2D), and positively with airway neutrophil absolute count (Rho = 0.50, $p = 0.01$) and percentage (Rho = 0.51, $p = 0.009$). These findings suggest a potential link between neutrophilic inflammation, airway macrophage PD-1 expression, and early structural lung damage in CF.

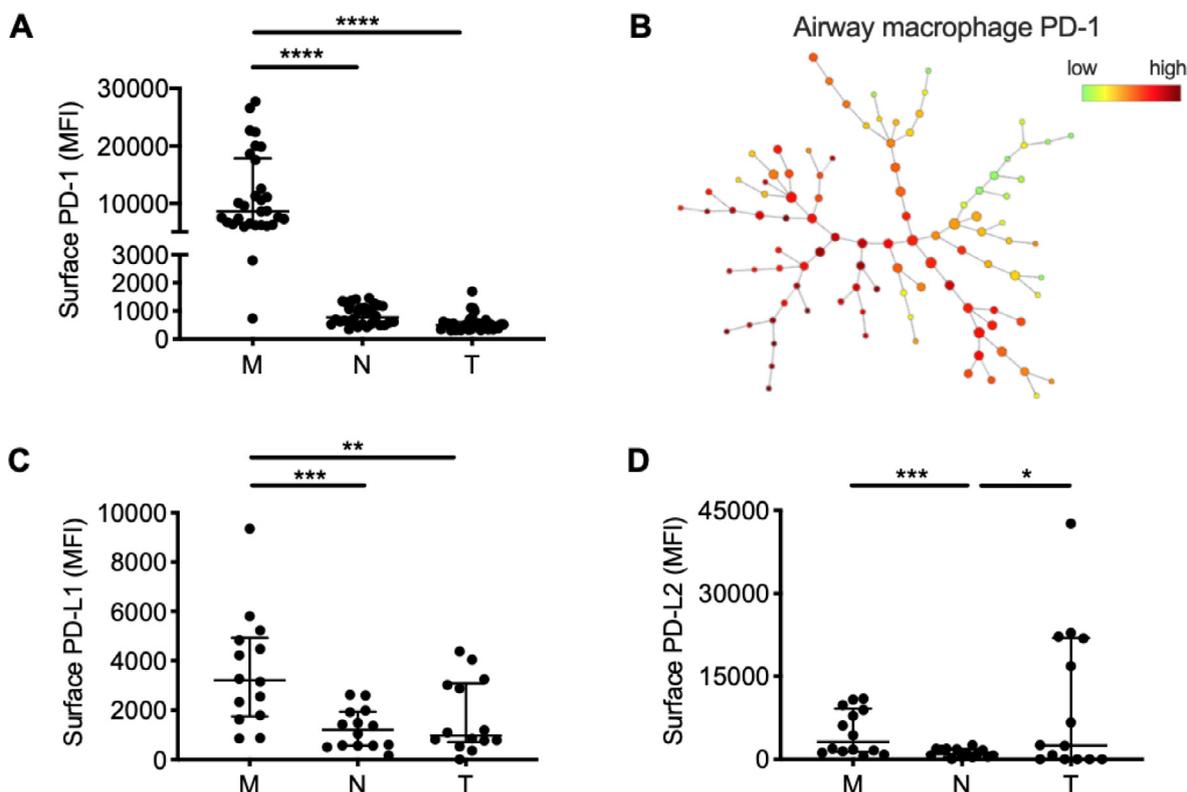


Fig. 1. PD-1 and its ligands, PD-L1 and PD-L2, are expressed in CF airway leukocytes. Flow cytometry analysis of BALF leukocytes from CF children shows higher PD-1 expression on airway macrophages (M) compared to neutrophils (N), and T cells (T) (A). SPADE analysis of airway macrophage PD-1 expression in different clusters (B). PD-L1 (C) and PD-L2 (D) expression in airway macrophages (M), neutrophils (N), and T cells (T) measured by flow cytometry shown as median fluorescence intensity (MFI). Data are shown as median and interquartile range, and analyzed by the Wilcoxon rank sum test, with significance levels indicated as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$.

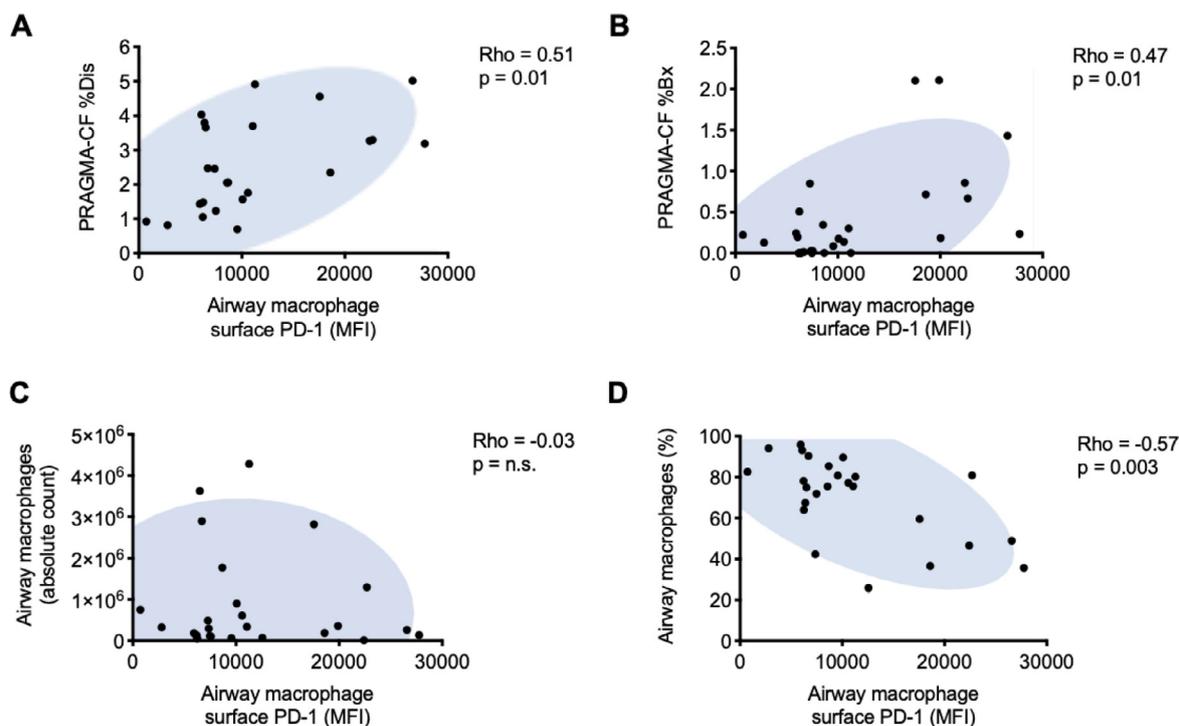


Fig. 2. PD-1 expression on airway macrophages correlates with lung disease in children with CF. PD-1 expression on CF airway macrophages is associated with PRAGMA-CF total disease score measured by CT (%Dis) (A) and with the bronchiectasis score (%Bx) (B). PD-1 expression on airway macrophages did not correlate with airway macrophage absolute count (C), but correlated negatively with airway macrophage percentage (D). Correlations were performed using the non-parametric Spearman's rank order correlation test and are shown as coefficient strength of Spearman Rho (left panel) and significance levels of p-values.

2.2. CFTR genotype and sex do not influence PD-1 expression in CF airway macrophages

Next, we investigated which intrinsic or extrinsic factors could contribute to PD-1 expression in airway CF macrophages. No significant difference was observed in the level of PD-1 expression based on sex (Fig. S2A) or between patients bearing F508Del homozygous vs compound heterozygous genotypes (Fig. S2B). Likewise, while uptake of oxidized lipids and the subsequent inability to efficiently metabolize them (lipidation index) correlates with a dysfunctional state in macrophages [27], as described in atherosclerosis [28] and several lung conditions including CF [29,30], in the subset of 22 children with CF for which Oil Red O staining was performed, no relationship was found between airway macrophage PD-1 expression and the lipidation index [31] (Fig. S2C).

Since lipidation, sex, and CFTR genotypes were not related to PD-1 expression on CF airway macrophages, we measured soluble and cell-bound inflammatory mediators in BALF and combined them with clinical variables in a bootstrap forest partitioning analysis (Table S3, N = 42 patients included). The main predictors for PD-1 expression on airway macrophages were patient age, neutrophil mediators, and infection with pro-inflammatory pathogens, defined by the presence of at least one of four pro-inflammatory pathogens, namely *P. aeruginosa*, *S. aureus*, *H. influenzae*, and *Aspergillus spp.* [32], at the time of BALF collection. While these factors may be related to each other, we recently showed that the presence of neutrophils in the airways of children with CF and their active exocytosis of NE-rich granules precedes colonization by pro-inflammatory pathogens, and that the extent of NE exocytosis did not differ between infected and non-infected groups at the single cell level [20]. However, LPS is an inducer of PD-1 expression on macrophages [14] and upon infection with pro-inflammatory pathogens, neutrophils are increasingly recruited to the airways, resulting in the extracellular accumulation of neutrophil-derived mediators. Therefore, to untangle the contribution of neutrophil and bacterial mediators in inducing PD-1 expression, we performed subsequent analyses discriminating between patients that presented with pro-inflammatory pathogens and those that were classified as not infected.

2.3. Neutrophil activation modulates PD-1 expression by CF airway macrophages

Considerable overlap was observed between infected and non-infected groups in inflammatory mediator levels, suggesting that a high state of immune activation is not exclusive to patients with airway infections (Fig. S3). We then assessed factors correlated with PD-1 expression on airway macrophages in the absence of infection (Fig. 3A). Significant correlations were observed for extracellular NE (Rho = 0.62, p = 0.02) and IL-8 (Rho = 0.48, p = 0.04), suggesting a role for neutrophil-derived soluble factors in the upregulation of PD-1 in airway macrophages.

To determine whether neutrophil-released mediators could directly modulate airway monocyte/macrophage surface PD-1 expression, we exploited an *in vitro* transmigration model previously used to recreate the CF airway neutrophil phenotype observed *in vivo* [18], which includes increased lifespan, active release of pro-inflammatory cytokines and exocytosis of granule enzymes such as NE and myeloperoxidase. Upon *in vitro* generation of CF airway neutrophils, media was conditioned with these cells and then subsequently applied to a blood monocyte transmigration model [33] (Fig. S4). Interestingly, monocytes that transmigrated into the conditioned media from CF airway-like neutrophils showed increased expression of surface PD-1 compared to both their blood counterpart and also to those monocytes that transmigrated into the transmigration control supernatant (conditioned

media from neutrophils transmigrated towards the leukotriene B4 - LTB4) (Fig. 3B). Our experimental model suggests soluble mediators from neutrophils recruited to CF airways could be directly modulating airway monocyte/macrophage PD-1 expression.

2.4. PD-1 increases uniformly on CF airway macrophages upon infection

Since the likelihood of infection increases with age and correlates with enhanced neutrophil recruitment to CF airways [34,35], we next fitted a logistic regression model for infection given age, and then a multivariate linear regression model for PD-1 expression on airway macrophages adjusted for age and infection. Unsurprisingly, we found that older children were more likely to harbor infections (p = 0.038) and that age was also a significant predictor of PD-1 expression (p < 0.001). However, even after adjusting for age, the effect of infection on PD-1 expression remained significant (p < 0.001). These findings support the notion that while age increases the risk of infection in CF, both age and infection can independently impact PD-1 expression on airway macrophages in children with CF.

Next, we investigated whether the increase of PD-1 expression on airway macrophages upon infection was confined to CF or if it could be applicable to age-matched controls. Therefore, we collected blood and BAL from 10 age-matched non-CF disease control children with aerodigestive diagnosis, prone to aspiration-induced inflammation, and analyzed the cells by flow cytometry. Similar to children with CF, airway macrophages showed higher PD-1 expression compared to airway neutrophils and T cells by 10- and 18-fold, respectively (Fig. S5A). Furthermore, we found that the two cohorts did not differ in their lipidation index (Fig. S5B), neutrophil percentage (Fig. S5C), or macrophage percentage (Fig. S5D). However, macrophage count was elevated in CF compared to non-CF disease control BALF, consistent with previous studies [36]. CF patients with the highest absolute macrophage counts tended to be younger (approximately 0.25–2 years) (Fig. S5E).

The significant increase in PD-1 expression on CF airway macrophages in the presence of infection compared to non-CF aerodigestive controls (Fig. 4A) suggests differential regulation of this pathway in CF airway disease. Clustering analysis showed that PD-1 expression was upregulated uniformly when comparing infected and non-infected subgroups (Fig. 4B), suggesting that infection with pro-inflammatory pathogens does not correlate with the modulation of PD-1 expression on any particular SPADE-defined subset of airway macrophages. Interestingly, among all surface markers included in our cytometry panel, PD-1 emerged as the most significant feature in the airway macrophage population discriminating between infected and non-infected children with CF (p < 0.0001, paired sample t-test). None of the other surface markers listed as significant discriminators between the two groups reproduced the uniform pattern observed with PD-1. Furthermore, differences in airway macrophage cell frequencies between the infected and not infected groups were observed (Fig. 4C). Out of the 100 clusters in the SPADE tree, 52 clusters, matching the lower PD-1 expression originally shown in Fig. 1B, were more prevalent in the non-infected group. Taken together, these results show that while certain airway macrophage subsets become more prevalent during infection, all airway macrophages upregulate surface PD-1 expression upon presence of pro-inflammatory pathogens.

2.5. PD-1 blockade in CF airway cells improves bacterial clearance

Next, we investigated whether presence of pro-inflammatory bacteria could modulate PD-1 expression on airway macrophages. To this end, we designed a short-term culture assay in which fresh BALF samples from CF children were collected (demographics in

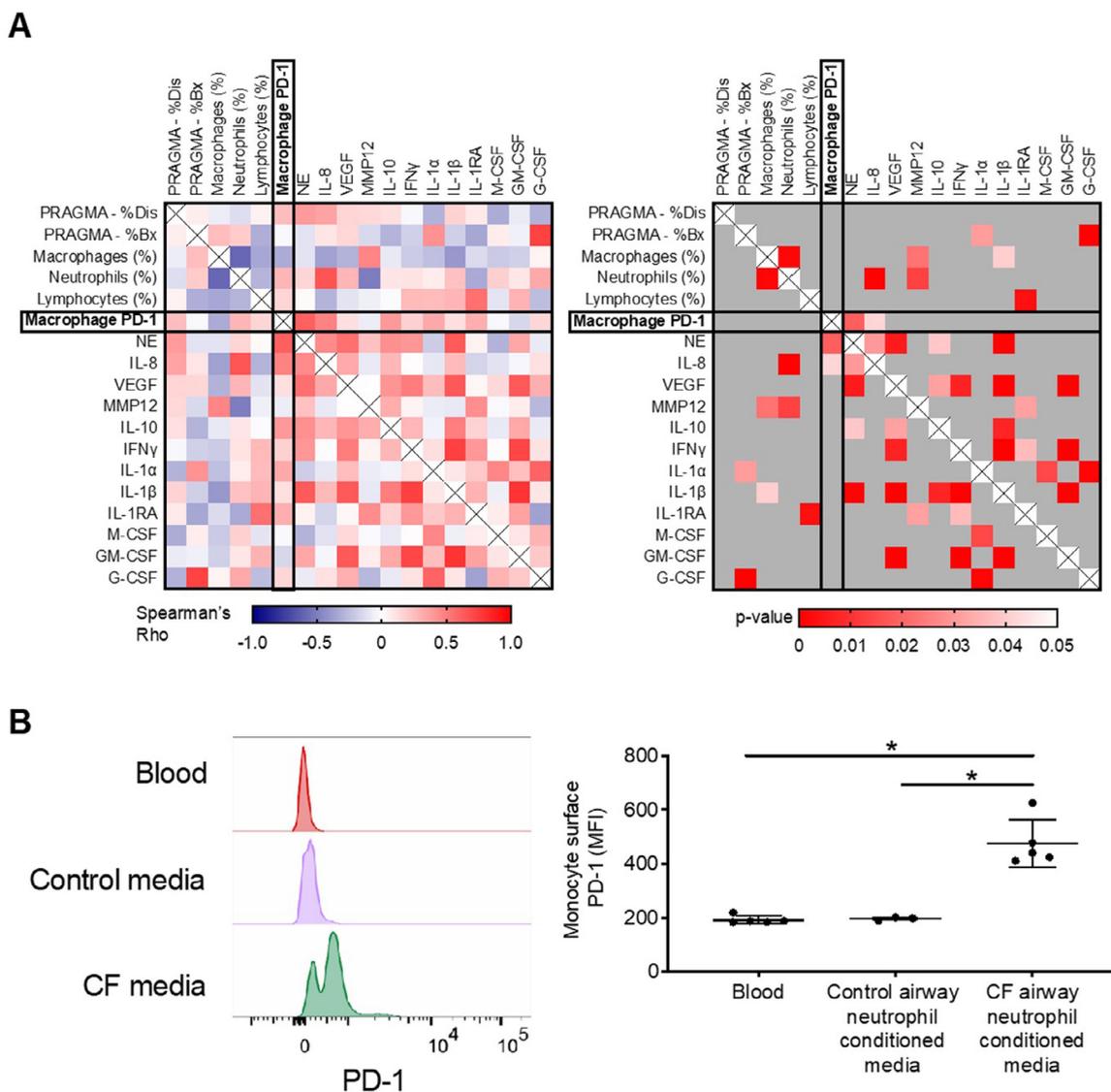


Fig. 3. PD-1 expression on CF airway macrophages is associated with neutrophilic inflammation. PD-1 expression on CF airway macrophages correlates with neutrophil-derived mediators in young children with CF with negative airway cultures. Correlations are shown as coefficient strength of Spearman Rho (left panel) and corresponding significance levels of p-values (right panel, with grey indicating $p > 0.05$) (A). *In vitro* transmigration of monocytes into neutrophil conditioned media (illustrated in Fig. S4) show modulation of PD-1 expression in monocytes exposed to the CF-like neutrophil conditioned media (B). Data are shown as median and interquartile range, and analyzed by the Wilcoxon rank sum test, with significance levels indicated as * for $p < 0.05$.

Table S3), minimally processed to remove endogenous bacteria and incubated with the pro-inflammatory pathogen *P. aeruginosa*. Co-incubation with bacteria did not change overall level of PD-1 expression on macrophages or the frequency of cells positive for PD-1 compared to cells not exposed to bacteria *in vitro* (Fig. 4D-E). To determine whether surface PD-1 on airway immune cells was active and influencing cell behavior we leveraged the same short-term culture assay and assessed bacterial killing in the presence or absence of PD-1 signaling blockade using a combination of a PD-1 blocking antibody and treatment with SHP099 (inhibitor of the PD-1-associated phosphatase SHP2). In a subset of 8 primary BALF samples from children with CF with sufficient leukocyte yield to conduct this assay, bacterial killing significantly improved upon PD1 blockade (Fig. 4F), suggesting that surface PD-1 is indeed active in airway leukocytes in CF and that its activity reduces bacterial clearance.

Finally, we investigated if PD-1 expression was dependent on CFTR function using our *in vitro* monocyte transmigration model (Fig. S4). Monocytes from healthy control (CFTR-competent)

donors were incubated with CFTR inhibitor 172 and monocytes from CF (CFTR-defective) donors were incubated with elxacaftor/tezacaftor/ivacaftor, then transmigrated to LTB4 or CFASN with the same drugs present. Neither treatment significantly impacted expression of PD-1 or PD-L1 in either transmigration condition, though some donor variability was observed (Fig. S6). Overall, our findings are consistent with the notion that the CF airway microenvironment, rather than intrinsic differences in CFTR function in monocytes/macrophages, leads to a change in poise in part linked to PD-1 signaling. This change is concomitant with early neutrophilic inflammation and a decrease in bacterial killing capacity, opening the path for chronic CF airway disease (Fig. 5).

3. Discussion

Immune responses in CF airways depend on close interactions between the CF epithelium, which shows enhanced pro-inflammatory signaling [37,38], resident macrophages and recruited neutrophils, mucus, and colonizing pathogens, which evolve over the course of the disease [39,40]. Macrophages and

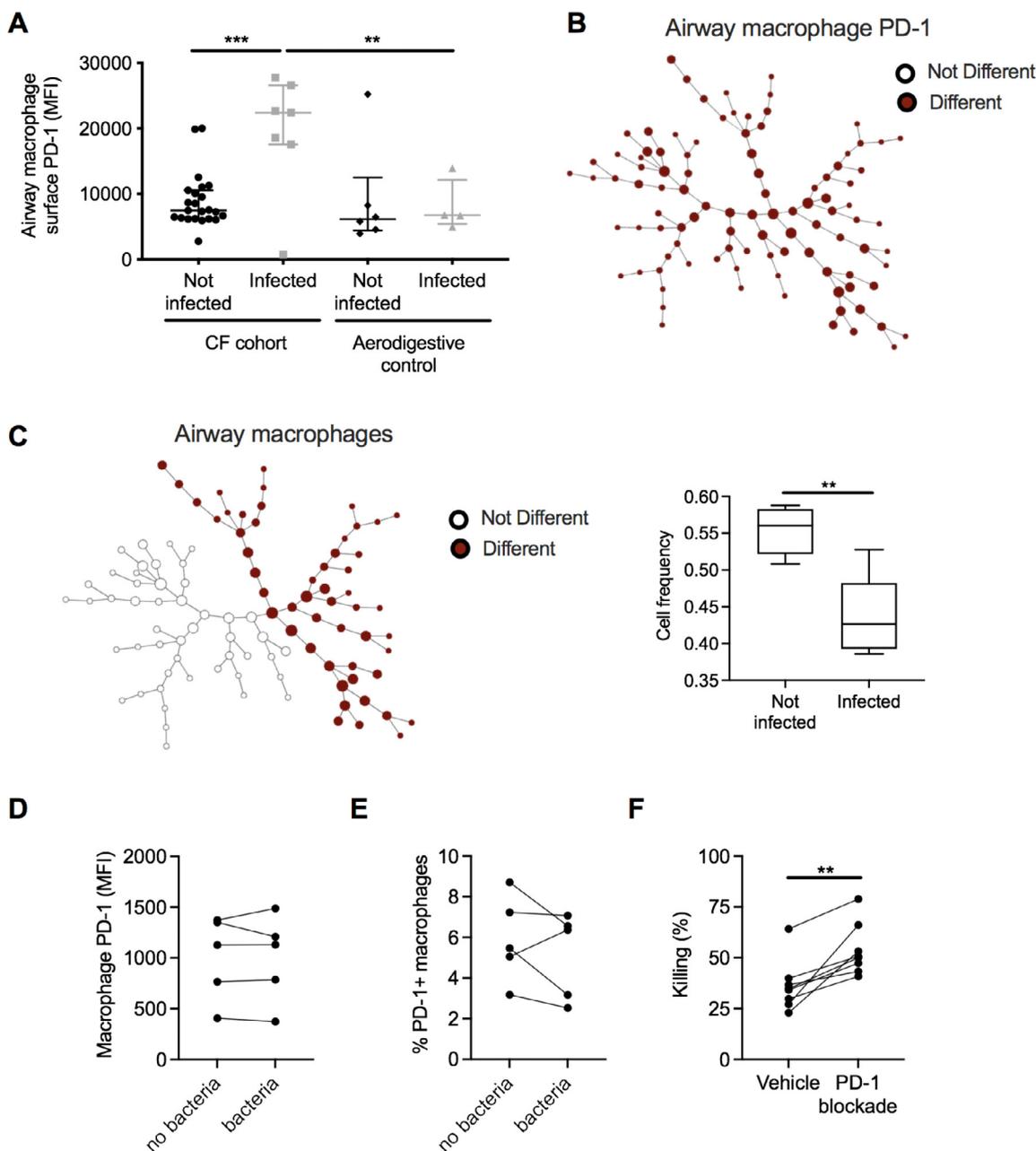


Fig. 4. PD-1 expression and function in CF airway macrophages associates with airway infection. Children with CF showed higher PD-1 expression on airway macrophages upon infection compared to non-CF disease control children (N = 22 and N = 10, respectively). Data are shown as median and interquartile range, and analyzed by the Wilcoxon rank sum test, with significance levels indicated as ** for p < 0.01, *** for p < 0.001 (A). SPADE analysis of CF airway macrophages shows that the difference in PD-1 expression levels measured between infected vs. non-infected CF children is due to uniform PD-1 modulation across all macrophage subsets (dark red) (B), and changes in frequency in defined cell clusters (C). Subset size reflects the number of macrophages with similar levels of marker expression. BALF leukocytes from eight young children with CF were treated *ex vivo* with anti-PD1 (antibody) combined with a SHP2 inhibitor (SHP099) while co-incubated with *P. aeruginosa* for 1 hour (D-F). Expression of PD-1 on macrophages and frequency of PD-1% macrophages was compared in samples with and without bacteria co-incubation. Subject-matched data points are connected and analyzed by the Wilcoxon rank sum test (N = 5, no differences). Killing was determined by CFU counts (N = 8). Treatment effectiveness was calculated by subtracting the effect of therapy on bacteria alone and killing capacity of the cells without treatment. Subject-matched data points are connected, and analyzed by the Wilcoxon rank sum test with significance levels indicated as ** for p < 0.01.

neutrophils in particular collaborate and modulate each other during responses [41,42], by dynamically sharing released factors such as NE [43], implementing bacterial clearance [44], and orchestrating gene expression [45]. Taken together, data presented here are consistent with a model in which airway macrophages in children with CF undergo apparent exhaustion, marked by increased PD-1 expression, which associates with neutrophilic inflammation and infection with pro-inflammatory pathogens, resulting in significant

lung damage. However, while at later stages of disease this phenomenon may be more widespread to the whole lung, in early CF lung disease, upregulation of PD-1 on airway macrophages likely occurs only in discrete diseased areas.

We recently showed that presence of a pathogenic neutrophil subset in CF airways, which actively exocytoses NE-rich granules, is detectable prior to the sustained presence of pro-inflammatory pathogens, and whose prevalence is not influenced by a pa-

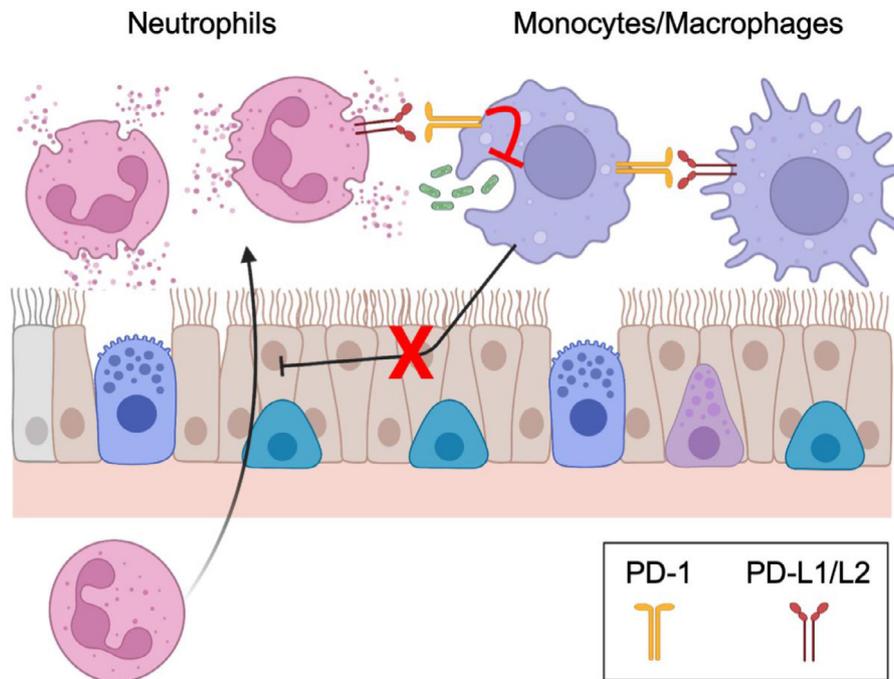


Fig. 5. Pathological model of PD-1 signaling in CF airway monocytes/macrophages. Increased PD-1 expression on CF monocytes/macrophages increases with age, and associates with chronic neutrophilic inflammation and hyperoxocytosis and bacterial infection in the airways.

tient's infection status [20]. Here, we showed that macrophage PD-1 expression was not directly modulated by presence of pro-inflammatory bacteria *in vitro*, but expression was elevated in children with CF who had detectable airway infection. Further, the differential expression of PD-1 observed between patients with CF and the control cohort could be due to epigenetic reprogramming induced by the local microenvironment [46]. Macrophages have previously been proposed as potential therapeutic targets for CF airway disease [47]. However, studies addressing the impact of new CFTR modulators and correctors have yet to fully address their influence on *intrinsic* defects of CF airway macrophages [48]. In that context, there is a clear need for the development of new therapeutic agents aiming at the direct modulation of *extrinsic* regulatory pathways in macrophages, which may reinvigorate pathogen clearance and delay neutrophil takeover seen in the early phase of pro-inflammatory pathogen infection and lung damage in children with CF. Here, we showed preliminary evidence that PD-1 blockade in airway leukocytes improved bacterial clearance. Because PD-1 is known to play a key role in controlling macrophage responsiveness in several conditions, modulation of PD-1 signaling may be of interest in airway diseases other than CF which also feature chronic bacterial infections [13,15,16].

Further studies are needed to address downstream effects of PD-1 signaling in CF airway macrophages, although prior evidence linked PD-1 signaling to changes in autophagy [49], which controls pathogen clearance by macrophages [50]. Moreover, while *P. aeruginosa* did not change PD-1 levels on macrophages, it may modulate macrophage responsiveness by altering expression of the PD-1 ligand and PD-L1, which would increase the likelihood of PD-1 inhibitory signaling in these cells [51]. While we did not observe a direct link between CFTR function and PD-1 expression on airway-conditioned monocytes *in vitro*, further studies are needed to determine if altered PD-1 signaling and/or intrinsic effects of CFTR deficiency in airway macrophages cause an inability to clear pathogens in CF children. While it has been previously shown that CFTR mutations can affect *in vitro* responses of monocyte-derived macrophages

[52], less is known about CFTR function in resident human airway macrophages.

3.1. Limitations of study

In this study we consider the transition from macrophage dominance to neutrophil dominance in the lung, but we do not define specific subsets of macrophage-lineage cells, such as inflammatory monocytes, monocyte-derived macrophages, and alveolar macrophages in BAL samples. Future studies implementing new techniques such as CITE-Seq are needed to pursue deeper investigation of CF airway monocyte/macrophage subsets revealed in our initial flow cytometry and SPADE analysis, with a particular focus on changes in PD-1 expression upon infection and neutrophil recruitment. Also, while this study shows a novel immunomodulatory pathway present in CF airway disease, it does not delineate whether the enhanced PD-1 expression on airway macrophages observed in patients with detectable infection is a cause or a consequence of the progressive neutrophil takeover. Indeed, a limitation in both this and prior [20] studies is that sensitive bacterial metagenomic analyses were not available, such that we cannot fully state that given patients were free of pro-inflammatory pathogens even if clinical microbiology cultures were negative. However, the *in vitro* modulation of PD-1 expression by neutrophil-derived factors did occur in the absence of pathogens or pathogen-derived molecules, suggesting a host-host interaction between CF airway neutrophils and monocytes/macrophages. Given limitations in BAL volume and cell number from pediatric subjects, we made a concerted effort to maximize use of each sample, but were not able to assess all possible targets of interest. One example is IFN α which has been reported to induce PD-1 expression in macrophages and should be assessed in future studies. Even though our *in vitro* data suggest little acute impact of CFTR modulator therapy on lung-recruited monocytes, it will also be interesting to assess lung macrophage PD-1 expression and poise in BAL

from infants with CF as CFTR modulator therapy becomes available to toddlers and infants.

4. Methods

4.1. Experimental model and subject details

4.1.1. Study visits

Prospective study visits were conducted on 45 CF children aged 3 to 62 months of age enrolled in AREST-CF (Perth, Australia), I-BALL (Rotterdam, The Netherlands) and IMPEDE-CF (Atlanta, GA, USA) early disease surveillance programs, and 10 age-matched non-CF controls undergoing bronchoscopy for clinical indications at the Aerodigestive Clinic at Children's Healthcare of Atlanta (Atlanta, GA, USA). The study was approved by relevant Institutional Ethical Review Boards at each site (1762/EP for AREST-CF; NL49725.078.14 for I-BALL; IRB#00097352 for IMPEDE-CF).

4.1.2. H441 cell line

H441 cells (ATCC, Cat# HTB-174) were cultured in DMEM/F-12 media supplemented with 10% FBS (Corning), 2mM glutamine (Sigma), and 100 U/mL-0.1mg/mL penicillin/streptomycin (Sigma). 2.5×10^5 H441 were harvested at passages 2-3 and cultured at air-liquid interphase on the Alvetex scaffold (Reprocell) coated with Rat tail collagen (Sigma) as previously described [53]. The basolateral medium DMEM/F-12 media supplemented with 2% Ultrosor G (Crescent Chemical Co), 2mM glutamine (Sigma) and 100 U/mL-0.1mg/mL penicillin/streptomycin (Sigma) was replaced every two days. After 14 days the membranes were inverted to allow neutrophil loading on the basolateral side and apical migration as described below.

4.2. Method details

4.2.1. Human sample collection and processing

Blood and BALF were collected from CF children (N = 45) and non-CF disease controls (N = 10). Demographic information for CF and non-CF disease control subjects is provided in **Table S1**. Blood was collected in K₂-EDTA tubes by venipuncture, cells and plasma were separated by centrifugation and the cellular fraction was used for flow cytometry. BALF was collected under general anesthesia using sterile saline as per the standard clinical procedure previously described [20]. BALF was then mechanically dissociated on ice in presence of 2.5 mM EDTA, cells were recovered after an 800 x g, 10-minute centrifugation at 4°C, then washed and used for downstream assays. BALF supernatant was further spun at 3,000 x g for 10 minutes at 4°C to remove debris and bacteria, and stored at -80°C until use. Differential cell counts and Oil Red O staining with lipid laden macrophage index [31] were performed by the clinical pathology laboratories at the Erasmus MC/Sophia Children's Hospital and Emory University/Children's Healthcare of Atlanta. Infection status was determined by clinical microbiology using standard aerobic culture techniques at each respective institution, and classified for presence of the pro-inflammatory pathogens *P. aeruginosa*, *S. aureus*, *H. influenzae*, and *Aspergillus spp.* [32]. Due to the nature of the sample collection process, the age of the patients, and cell yield, it was not possible to perform all assays on all collected samples. A detailed tally describing the assays performed on each sample is provided in **Table S2**.

4.2.2. Chest-computed tomography (CT) scans

Patients underwent a chest CT-scan without anesthesia using a Siemens SOMATOM® Force ultra-fast scanner at the Erasmus MC/Sophia Children's Hospital or under general anesthesia at the Perth Children's Hospital. The CT-images were scored for structural

lung damage using the Perth-Rotterdam Annotated Grid Morphometric Analysis for Cystic Fibrosis (PRAGMA-CF) scoring method [54]. Repeatability of PRAGMA-CF score has been previously described. Disease score (%Dis) was the total lung volume proportion of bronchiectasis, mucus plugging, and bronchial wall thickening.

4.2.3. Flow cytometry and SPADE analysis

Multiparametric flow cytometry analysis of whole blood and BALF cells was standardized across study sites by integrating two stringent calibration steps prior and post- sample acquisition using a fluorescent bead-based method (Biolegend), as previously illustrated [20] which provide constant and robust output from the flow cytometers, and used premixes of the antibodies listed above to stain blood and BAL cells. Samples were pre-stained for 10 minutes on ice in the dark with the Human TruStain FcX Fc blocking solution and the Zombie Aqua reagent (Biolegend). In addition, we also check for specificity of antibody staining with fluorescence-minus one controls, as previously explained [55]. The following staining was subsequently added on ice in the dark for 30 minutes: anti-human CD3 (pan-T cells), CD45 (pan-leukocytes), CD63, PD-1, PD-L1, PD-L2, CD66b, CD33, CD41a (all from Biolegend), CD66a (Novus Biologicals) and Siglec-8 (R&D Systems). Cells were washed, fixed in Lyse/Fix Phosflow (BD Biosciences) and acquired on a FACS Fortessa or LSRII (BD Biosciences). Analysis and compensation were performed in FlowJo V9.9.5 (FlowJo, LLC). Macrophages, neutrophils, and T cells were gated from blood and airway samples as detailed in **Fig. S1**. While gating of blood leukocyte populations is straightforward, gating of airway leukocytes is more challenging. Our gating strategy was devised to identify all monocyte/macrophage-lineage cells but was not sufficient to identify subsets. CD45 is a pan-leukocyte marker and was used to exclude epithelial cells and debris. CD3 was used to separate T cells from myeloid cells, which were separated into monocyte/macrophage and neutrophil populations using CD33 [56]. Clustering analysis and group comparisons were performed in SPADE V4.0 [26] using the following parameters: arcsinh transformation cofactor (150), neighborhood size (5), local density approximation factor (1.5), maximum allowable cells in pooled downsampled data (50,000), target density with fixed number of cells remain (20,000), K-means algorithm with 100 clusters.

4.2.4. Assays for soluble mediators in BALF

BALF cytokines were quantified using a U-PLEX multiplex chemiluminescent ELISA assay (Meso Scale Diagnostics), per manufacturer's protocol. *In vitro* enzymatic activity assays for NE and MMP12 were performed using Förster resonance energy transfer probes NEmo-1 and LaRee-5 respectively (Sirius Fine Chemicals SiChem GmbH), as described [57].

4.2.5. *In vitro* transmigration

Blood neutrophils and monocytes were isolated from healthy donors using Polymorphprep (Accurate Chemical) and Rosette-Sep (STEMCELL technologies) with Ficoll (GE Healthcare) kits, respectively, per manufacturers' protocols. Isolated blood neutrophils were resuspended in RPMI (Corning), loaded on the wells and transmigrated towards the chemoattractant control leukotriene B4 (LTB4, 100 nM, Sigma) or CF airway supernatant (CFASN, devoid of cells and bacteria), as we have detailed previously [18]. Neutrophils transmigrated through the epithelium to either LTB4 (transmigration control) or CFASN (CF airway-like condition) were collected after 4 hours, washed three times, and cultured in fresh RPMI for 8 hours at 37°C to generate conditioned media. These conditioned media were further purified by sequential centrifugations at 800 x g for 10 minutes, followed by 3,000 x g for 10 minutes. Isolated blood monocytes were then transmigrated in turn through the epithelium into the neutrophil-derived conditioned media in

new transmigration chambers for 5 hours and analyzed by flow cytometry as detailed above, with the replacement of anti-human CD33 by anti-human CD115 (Biolegend). All transigrations were performed in an incubator with 5% CO₂ at 37°C. Monocytes from CF donors were isolated using the same procedure, with subject demographics summarized in **Table S4**.

4.2.6. Ex vivo bacterial killing assay

Overnight cultures of the pro-inflammatory bacteria *Pseudomonas aeruginosa* (strain PAO1) were subaliquoted and grown to reach the exponential growth phase. The bacteria were then incubated in RPMI, supplemented with 10% FBS (Corning), on an end-over-end rotating wheel for 30 minutes at 37°C. Fresh BALF leukocytes were resuspended in RPMI, 10% FBS and incubated at 37°C, 5% CO₂ for 15 minutes. Co-incubation of bacteria and immune cells, in presence or absence of PD-1 blockade, was performed at a multiplicity of infection of 1, in RPMI, 10% FBS, on an end-over-end rotating wheel for 1 hour at 37°C, 5% CO₂. PD-1 blockade was accomplished using 1 µg/mL of Ultra-LEAF purified anti-human PD-1 blocking antibody (Biolegend) combined with 80 nM of SHP099 (Cayman Chemical), a small molecule SHP2 inhibitor [58]. Total bacterial killing was assessed by gently lysing the cells in 0.1% Triton-X and plating of bacteria on an agar plate overnight at 37°C. Bacterial killing capacity was calculated using colony forming units (CFU), with the bacteria, RPMI, 10% FBS condition set as 100% survival. Treatment efficacy was calculated by subtracting the effect of PD-1 blockade on bacteria alone and the killing capacity of immune cells without treatment from the condition where bacteria, immune cells, and PD-1 blockade were all present. Demographic information for CF subjects used for the bacteria killing assays is summarized in **Table S4**.

4.3. Quantification and statistical analysis

4.3.1. Statistical analysis

Data were compiled in Excel (Microsoft) and transferred to JMP13 (SAS Institute) and Prism v7 (GraphPad) for statistical analysis and graphing, respectively. The effect of study site on measured variables was corrected, while adjusting for age using a non-parametric empirical Bayesian method as previously described [20]. Predictor screening analysis was performed using bootstrap forest partitioning in JMP13, while simple linear fitting modelling was performed in R. Correlations were calculated using Spearman's rank-order correlation, a non-parametric rank-based calculation that is relatively insensitive to outlier effect.

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Author Contributions

All authors read, reviewed and agreed to submit the manuscript. Study conception: S.M.S., R.T., H.M.J.; Study implementation: C.M., H.H., L.W.G., V.D.G., C.S., L.G.; Supervision of statistical analyses: L.P.; Unrestricted access to all data: H.H., L.G., R.T., C.M. H.M.J.; Critical technical and/or logistical support: A.S.D., T.R., B.S.D., H.S.L., D.L.F. M.V., G.L.S., M.R.B., C.S., H.A.W.M.T., S.R., J.D.C., P.Q., L.P., B.J.S., M.A.M., A.K., L.G.; Manuscript writing: C.M., R.T., V.D.G.; Manuscript review: H.H., L.W.G., T.R., B.J.S., J.D.C., B.J.S., M.A.M., A.K., L.G., S.M.S., H.M.J.

Declaration of Competing Interest

None.

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Supplementary materials

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