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Neutrophils in idiopathic pulmonary fibrosis patients are phenotypically distinct to controls

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Abstract:	

Neutrophils in idiopathic pulmonary fibrosis patients are phenotypically distinct to controls

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Take home message

Neutrophils in idiopathic pulmonary fibrosis (IPF) patients exhibit functional distinctions, suggesting a mechanism for their recruitment into the lungs. This study underscores the need for further research into the role of neutrophils in the progression of IPF.

To the Editor:

Neutrophils, key immune cells, are increased in the air-spaces of patients with idiopathic pulmonary fibrosis (IPF) and serve as an independent prognostic biomarker [1, 2]. Recent studies show that blood neutrophil-to-lymphocyte ratios (NLR) correlate with IPF lung function and predict outcome [3]. Neutrophils may contribute to fibrotic lung disease via the production of reactive oxygen species (ROS), proteinases (e.g. neutrophil elastase), and neutrophil extracellular traps (NETs) [4-7]. However, the precise mechanisms by which neutrophils are recruited and retained within the lung to drive IPF disease progression remain unclear.

Transcriptomic studies highlight that neutrophils are a heterogenous population, with distinct functional subsets occurring during disease [8, 9]. However, no consensus exists regarding specific surface markers for neutrophil sub-types. A subset of mature neutrophils, defined as $CD16^{bri}CD62L^{dim}CD11b^{+}$, has been demonstrated to have enhanced ROS production, whilst suppressing T cell responses [10]. Whether neutrophil functionality or surface marker expression is altered in IPF is unknown.

In this observational study, we investigated neutrophil functionality and surface marker expression in a small cohort of patients with IPF (*n*=5-26) and asymptomatic non-interstitial lung disease (non-ILD) controls (*n*=4-16) that were consecutively recruited at the national ILD Service at University College London Hospital between May 2017 and January 2020. Written informed consent was obtained from all participants (UK NHS IRAS study I.D. 29531). All IPF patients were diagnosed based on clinico-radiological multidisciplinary team review. Non-ILD controls included individuals with haemoptysis (31%), COPD/bronchiectasis (32%), abnormal chest x-ray scans (25%), asthma (6%) or emphysema (6%). The percentage of non-ILD peripheral neutrophils was comparable with healthy controls (data not shown), to indicate these non-ILD controls did not have systemic inflammation at time of recruitment. In the non-ILD cohort, the mean age was 64±13 years, 50% male, with an average neutrophil blood count of 4.94±2.13 $x10^9$/L. Patients with IPF were significantly older compared to the non-ILD cohort at 76±8 years (*p*=0.001), and pre-dominantly male (80%) with an average neutrophil blood count of 5.94±2.03 $x10^9$/L, which was not significantly different compared to non-ILD controls (*p*=0.17).

To investigate neutrophil functionality, blood and broncho-alveolar lavage fluid (BALF) was collected from patients and neutrophils isolated for neutrophil chemotaxis studies as described [2]. To assess surface marker expression on isolated peripheral blood or BALF neutrophils, flow cytometric analysis (figure 1a) was performed using anti-CD11c-FITC, anti-CD54-APC, anti-CD11b-V450, anti-CD18-FITC, anti-CD16-APC-Cy7 and anti-CD62L-PE fluorescently-conjugated antibodies (all from BD Biosciences, USA) in separate cohorts of patients with IPF or non-ILD controls. In later experiments, an anti-CXCR1-PerCP-Cy5.5 antibody (BD Biosciences) was added to the staining panel. Flow cytometric data was analysed using FlowJo v10.10 software (BD Biosciences). Two-way ANOVA was performed, with Holm-Sidak multiple comparisons post-hoc testing, using GraphPad Prism v10.4.1 software (GraphPad, USA).

Flow cytometric analysis revealed that peripheral neutrophils from IPF (*n*=24) and non-ILD control patients (*n*=15) have equivalent expression of CD11c, a neutrophil maturation marker (figure 1b, *p*=0.987) suggesting unaltered maturation. However, expression of CD16, another neutrophil maturation marker, was significantly reduced on peripheral IPF neutrophils compared to non-ILD controls (figure 1b, *p*=0.001). Expression of CD62L, a cell adhesion molecule, was comparable between the two patient cohorts (figure 1b, *p*=0.986). Furthermore, we found that CXCR1, a chemokine receptor, was significantly up-regulated in IPF neutrophils compared to controls (figure 1b, 35,305±10,348 vs. 15,122±3,067 MFI units respectively, *p*=0.005). IPF neutrophils also showed a two-fold increase in chemotaxis towards N-formyl methionyl-leucyl-phenylalanine (fMLP), a well-described neutrophil chemoattractant, compared to controls (figure 1c, *p*=0.002). It is known that increased secretion of neutrophil chemokines including CXCL8, which has high affinity for CXCR1,

is found in the lungs and airways of patients with IPF compared to controls [11]. Therefore, increased expression of CXCR1 on peripheral neutrophils may allow for increased trafficking towards chemokines expressed in the lung during IPF.

Patients with IPF have significantly more neutrophils in their BALF than non-ILD controls (44.7±12.5 vs. 22.4±8.2 % of total cells respectively, *p*=0.016). Further analysis also showed an increased frequency of BALF neutrophils expressing more CD18 (86.6±14.4 vs. 53.9±16.9 % respectively, *p*=0.014. 3,344±1,894 vs. 1,934±1,058 MFI units respectively) and CD11b (87.9±9.9 vs. 60.0±27.0 % respectively, *p*=0.012. 2,121±1,402 vs. 1,402±363 MFI units respectively) in IPF compared to non-ILD controls (figure 1d). Additionally, fewer IPF BALF neutrophils expressed CD54 (ICAM-1), a marker for reverse transmigration, than control neutrophils (figure 1d, *p*=0.042, MFI = 4,226±2,911 vs. 21,486±12,123 respectively). We also found a trend of decreased ICAM-1 expression on peripheral neutrophils from IPF patients compared to non-ILD controls, but this did not reach statistical significance (*p*=0.205, data not shown). Reverse transmigration is a process by which neutrophils exit inflamed tissues and return back into the circulation for removal by apoptosis in the bone marrow and is characterised by high ICAM-1 expression [12]. Our data suggests that neutrophils in IPF patients may be retained in the lung and are not reverse transmigrating back into the circulation.

Lastly, given that CD16 expression was significantly altered on peripheral neutrophils from IPF patients, we explored the expression of other surface markers, CD62L and CD11b, on CD16 expressing neutrophils. Whilst the percentage of $CD16^{bri}CD62L^{dim}CD11b^{+}$ neutrophils was similar in the blood of patients with IPF (5.9±16.1 vs. 4.2±5.1 % respectively) compared to controls (figure 1e, *p*=0.934), there was a significantly increased frequency of $CD16^{bri}CD62L^{dim}CD11b^{+}$ neutrophils in the BALF of patients with IPF than controls (figure 1e, 25.4±22.4 vs. 4.6±6.5 % respectively, *p*=0.008). This indicates that neutrophils have differential surface marker expression patterns in different cellular compartments of IPF patients.

Overall, our findings demonstrate that neutrophils from patients with IPF exhibit distinct phenotypic and functional characteristics compared to non-ILD controls, with increased expression of adhesion molecules, enhanced chemotactic responses towards fMLP and reduced ICAM-1 expression. This suggests that in IPF, there may be impaired clearance of neutrophils from the lung. However, more studies are needed to explore FPR1 expression, the receptor for fMLP, and chemotaxis towards IL-8, the ligand for CXCR1, in IPF neutrophils. It is also still unclear whether enhanced neutrophil retention in the lung drives fibrotic tissue damage. Furthermore, the increase in BALF $CD16^{bri}CD62L^{dim}CD11b^{+}$ neutrophils in IPF patients, a subset associated with enhanced ROS

production, highlights the need for further research into their role in driving fibrotic disease progression.

This study shows that neutrophils can adopt different phenotypic traits during lung disease. Studies in other chronic lung diseases indicate increased circulating numbers of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), which correlate with poor outcome in patients with lung cancer [13]. Moreover, aged neutrophils, defined as $CD11b^{hi}CD62L^{lo}CXCR4^{+}$, are associated with increased NET production and fibrotic disease progression in a murine model [14, 15]. Further research will elucidate the specific mechanistic contributions of neutrophil subsets in IPF and whether they correlate with disease outcome.

This study does have some limitations, including small patient sample size. Additionally, our non-ILD control group consisted of a heterogenous group of patients, some of whom have chronic lung diseases, associated with neutrophil-driven inflammation. However, it was not possible to access BALF from healthy controls as a comparator group to study neutrophil phenotype under our study ethics. Furthermore, despite including patients with other respiratory diseases, we still found differences between IPF and non-ILD controls and this may be due to different neutrophil function in these chronic lung diseases (e.g. inflammatory vs. pro-fibrotic).

In summary, our findings indicate that neutrophil phenotype and function are modulated in IPF, and suggest a potential mechanism for their role in fibrotic disease progression. Further detailed exploration into the role of neutrophils in fibrotic lung disease may pave the way for the development of novel therapeutic strategies to mitigate neutrophil-driven fibrotic lung damage.

Financial support statement

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Figure legend

Figure 1: Neutrophils from IPF patients exhibit phenotypic and functional differences compared to non-ILD controls.

a) Representative flow cytometric gating strategy assessing surface marker expression on peripheral or BALF neutrophils from IPF or non-ILD control patients. Granulocytes were identified based on FSC vs. SSC parameters (i) and doublet exclusion (ii). (iii) Expression of CD11c, CD16, CD62L, CXCR1, CD11b, CD18 and CD54 (ICAM-1) on gated neutrophils (black histograms) was defined based on isotype control staining (open histograms). Lastly, expression of CD16 vs. CD62L (iv) was investigated on neutrophils, along with CD11b expression on the $CD16^{bri}CD62L^{dim}$subset (v).

b) Median fluorescence intensity (MFI) of CD11c, CD16, CD62L or CXCR1 expression on peripheral blood granulocytes from IPF (*n*=5-24 respectively) or non-ILD (*n*=4-15 respectively) patients.

c) Chemotaxis index of peripheral blood neutrophils (Φ) from IPF (*n*=7) or non-ILD control (*n*=6) patients towards fMLP (100 nM), as normalised to media controls.

d) Percentage of broncho-alveolar lavage fluid (BALF) neutrophils expressing CD18, CD11b or CD54 (ICAM-1) from IPF (*n*=6) or non-ILD (*n*=7) patients.

e) Percentage of $CD16^{bri}CD62L^{dim}CD11b^{+}$ neutrophils of total granulocytes in peripheral blood or BALF from IPF (*n*=26/6 respectively) or non-ILD (*n*=16/8 respectively) patients.

Two-way ANOVA was performed, with Holm-Sidak post-hoc testing (ns=not significant, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

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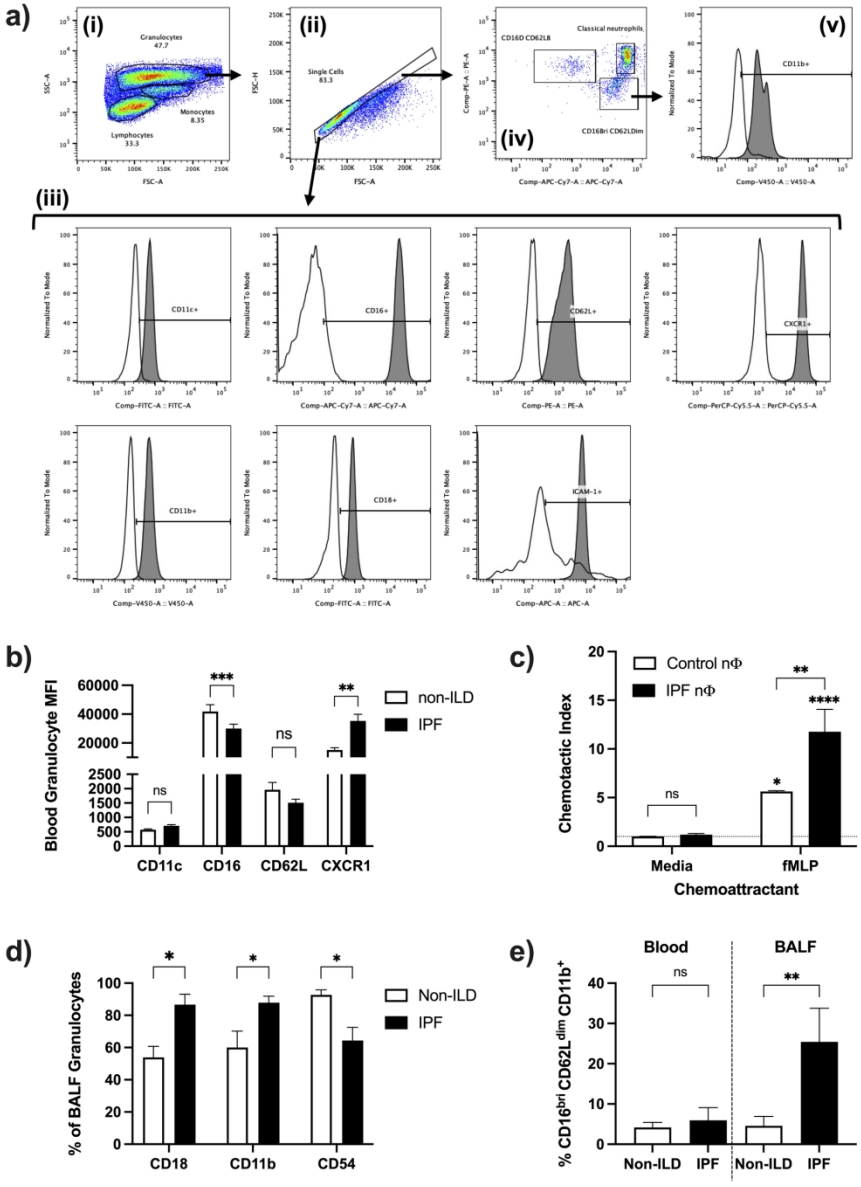

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(n=16/8 respectively) patients. Two-way ANOVA was performed, with Holm-Sidak post-hoc testing (ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

180x238mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	-	Due to restriction in characters length for the title, it was not possible to include the study design type.
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	N/A	N/A
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	1	Lines 21-35
Objectives	3	State specific objectives, including any pre-specified hypotheses	2	Lines 37-40
Methods				
Study design	4	Present key elements of study design early in the paper	2	Lines 37-40
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	2	Lines 37-40
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	2	Lines 37-40 & 41-42
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed	2	Lines 41-45
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	2	Lines 41-48
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	2	Lines 50-55
Bias	9	Describe any efforts to address potential sources of bias	2	Lines 45-48
Study size	10	Explain how the study size was arrived at	-	This was a small pilot study, therefore power calculations were unavailable to determine study size.

Continued on next page

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	2	Lines 50-56
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	2	Lines 56-57
		(*b*) Describe any methods used to examine subgroups and interactions	N/A	N/A
		(*c*) Explain how missing data were addressed	2	Lines 52-56. Separate patient cohorts were used to measure different flow cytometric parameters. Therefore different sample sizes are indicated for each measurement in the results.
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed	N/A	N/A
		(*e*) Describe any sensitivity analyses	N/A	N/A
Results				
Participants	13*	(a) Report numbers of individuals at each stage of study—e.g. numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	2-3	Lines 60-85. Sample size is also shown in the figure legend (lines 125-132).
		(b) Give reasons for non-participation at each stage	N/A	N/A
		(c) Consider use of a flow diagram	N/A	N/A
Descriptive data	14*	(a) Give characteristics of study participants (e.g. demographic, clinical, social) and information on exposures and potential confounders	2	Lines 44-48
		(b) Indicate number of participants with missing data for each variable of interest	N/A	N/A
		(c) *Cohort study*—Summarise follow-up time (e.g. average and total amount)	N/A	N/A
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	N/A	N/A
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g. 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N/A	N/A – due to small sample size in this pilot study, adjusted estimates was deemed not appropriate.
		(*b*) Report category boundaries when continuous variables were categorized	N/A	N/A
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A	N/A

Other analyses	17	Report other analyses done—e.g. analyses of subgroups and interactions, and sensitivity analyses	N/A	N/A
Discussion				
Key results	18	Summarise key results with reference to study objectives	3	Lines 87-89
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	3	Lines 104-107
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	3	Lines 96-102
Generalisability	21	Discuss the generalisability (external validity) of the study results	-	-
Other information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	4	Lines 116-119

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.