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Circulating dendritic cells are severely decreased in idiopathic pulmonary fibrosis with a potential value for prognosis prediction

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ABSTRACT

Dendritic cells (DCs) accumulate in the lung of patients affected by idiopathic pulmonary fibrosis (IPF). We measured the frequencies of circulating conventional CD1c + and CD141 + cells (namely, cDC2 and cDC1) and of plasmacytoid CD303 + DCs in a cohort of 60 therapy naive IPF patients by flow cytometry. Peripheral levels of reactive oxygen species (ROS) and of pro-inflammatory and Th1/Th2 polarizing cytokines were also analyzed. All blood DC subtypes were significantly reduced in IPF patients in comparison to age- and sex-matched controls, while ROS and interleukin (IL-6) levels were augmented. IL-6 expression increased along with disease severity, according to the gender-age-physiology index, and correlated with the frequency of cDC2. IL-6 and cDC2 were not influenced by anti-fibrotic therapies but were associated with a reduced survival, the latter being an independent predictive biomarker of worse prognosis. Deciphering the role of DCs in IPF might provide information on disease pathogenesis and clinical behavior.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating fatal lung disease with a mean survival of 2–5 years from diagnosis that generally remains refractory to treatment [1]. The estimated incidence of IPF is constantly growing ranging from 2.8 to 19 cases per 100.000 people per year in Europe and North America, respectively [2]. Disease behavior is highly variable with associated comorbidities exerting a significant impact on prognosis [3,4].

Pathogenesis of IPF has not yet been clearly elucidated. No more considered as an inflammatory disorder, IPF likely results from recurrent episodes of alveolar injury that promotes a pro-fibrotic microenvironment through an altered cross-talk between alveolar epithelial cells and resident fibroblasts [5,6]. Oxidative stress plays a key role in this scenario [7,8], as an oxidant/antioxidant imbalance has been reported in the serum and in the bronchoalveolar lavage (BAL) of IPF patients [9,10]. Increased levels of oxidative burst (OB) are crucial not only in tissue damage/remodeling but also in orchestrating the immune response through the modulation of both effector and regulatory T cells [11,12]. In addition, OB may also act by increasing the expression of pro-inflammatory and T-helper (Th)-2 polarizing cytokines, such as respectively interleukin (IL)-6 and IL-4 [13–15].

Dendritic cells (DCs) are professional antigen (Ag) presenting cells that exert an important role in immune surveillance in the lungs where they are strategically placed within the airway epithelium [16]. DCs originate from progenitor cells in the bone marrow, circulate in blood and are found in lymphoid organs, and in all tissues. In humans, blood DC subtypes include $CD11c^+$ conventional DCs (cDCs), that are $CD1c^+$ or $CD141^+$ cells, and $CD11c^-$ plasmacytoid DCs (pDCs), including $CD123^+$ or $CD303^+$ cells [17–20]. Conventional DCs, previously

Abbreviations: DCs, dendritic cells; **IPF**, idiopathic pulmonary fibrosis; **cDCs**, conventional dendritic cells; **ROS**, reactive oxygen species; **pDCs**, plasmacytoid dendritic cells; **OB**, oxidative burst; **Th**, T-helper; **IL**, interleukin; **Ag**, antigen; **BAL**, bronchoalveolar lavage; **mDCs**, myeloid dendritic cells; **Flt3L**, FMS-like tyrosine kinase-3ligand; **GAP**, gender-age-physiology; **WBCs**, white blood cells; **Hb**, haemoglobin; **$DLCO_{sb}$**, single-breath diffusing lung capacity of the carbon monoxide; **6-MWT**, 6-minute walk test; **DHR**, Di-Hydro-Rhodamine 123; **MFI**, mean fluorescence intensity; **TNF**, tumor necrosis factor; **INF**, interferon; **SD**, standard deviation; **ROC**, receiver operating characteristic; **OS**, overall survival; **FU**, follow-up; **HRs**, hazard ratios; **CI**, confidence interval; **CCL**, chemokine (C—C Motif) ligand; **CXCL**, C-X-C motif chemokine ligand; **AS DCs**, AXL^+ $SIGLEC6^+$ DCs; **BMI**, body mass index

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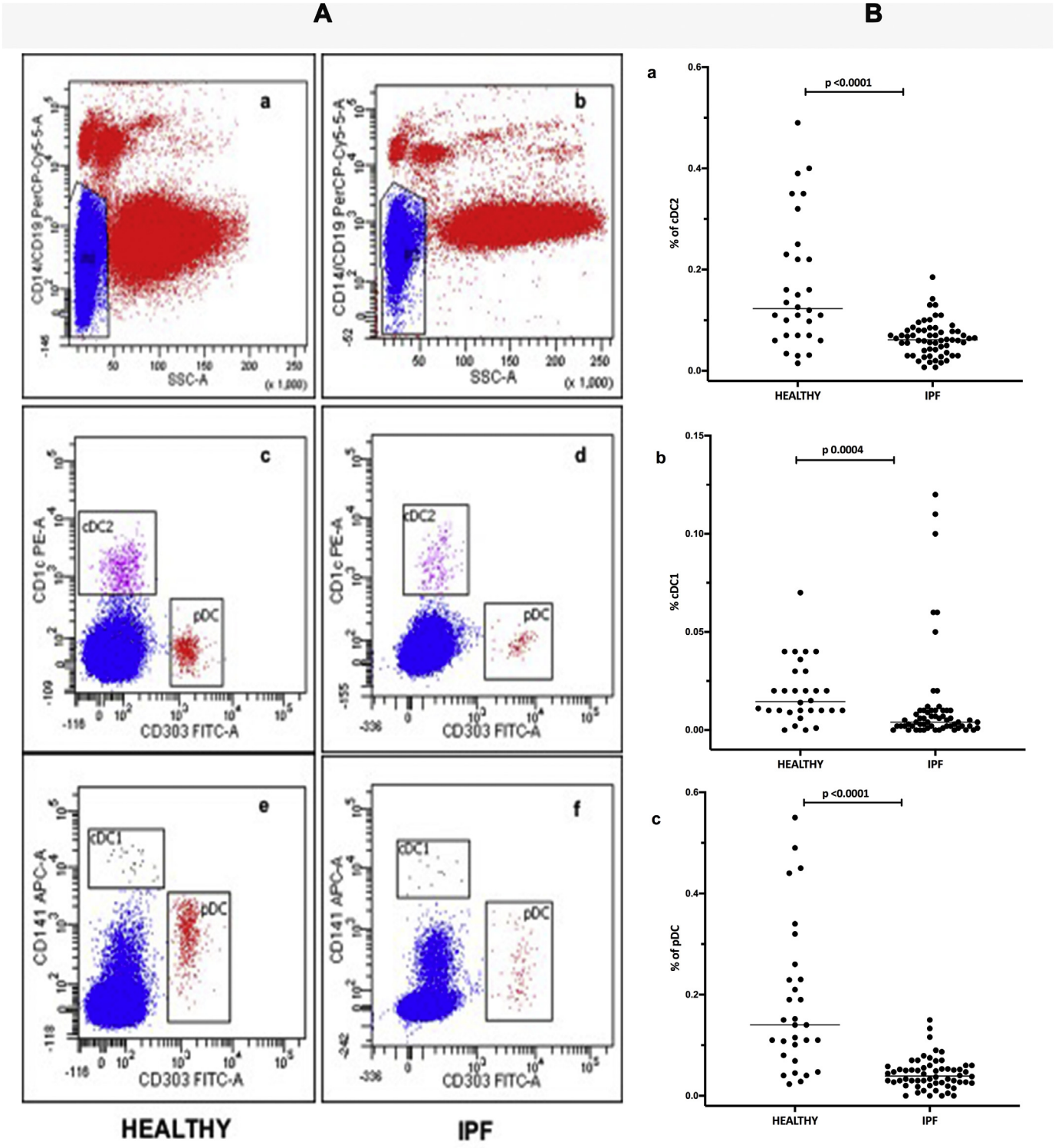

Fig. 1. A) Gating strategy for dendritic cell subsets identification.Total leukocytes were plotted according to SSC and CD19/CD14 expression (panels a and d). Low SSC and not CD19/CD14 expressing cells were selected in order to exclude monocytes, B cells and granulocytes. Selected cells were plotted according to CD303 and CD1c expression (panels c and e) to identify respectively plasmacytoid CDs and conventional DC2s. Further plotting according to CD303 and CD141 allowed the identification of conventional DC1s. B) Peripheral circulating dendritic cells are markedly reduced in idiopathic pulmonary fibrosis patients. Scatter plots showing the percentage distribution of circulating cDC2 (panel a), cDC1 (panel b), and pDC (panel c) subsets among total leukocytes in healthy subjects and IPF patients.

termed type-1 ($CD1c^+$) and type-2 ($CD141^+$) myeloid DCs (mDCs), have recently reclassified as cDC2 and cDC1, respectively [17].Conventional DCs exert a key function ranging from pathogen detection to cancer immunity as they are critical, through antigen presentation, for the initiation of specific T-cell responses. On the other, pDCs display high anti-viral activities due to the ability to produce type I interferon and are thought to be involved in immune tolerance [18,19].

Recent evidence suggest that DCs are likely involved in the pathogenesis of IPF. Immature DCs have been shown to accumulate in response to cytokines and transcription factors, including the DC-specific

growth factor FMS-like tyrosine kinase-3 ligand (Flt3L), in human fibrotic interstitial lung diseases [20]. In line with this finding, Flt3L has been found to be upregulated in the serum and lung tissue of IPF patients likely contributing to an increased numbers of $CD11b^+$ DCs in the fibrotic areas [21]. In a previous report, mature DCs along with non-proliferating B and T lymphocytes have been shown to create ectopic organized lymphoid structures in the lung of IPF patients, along with increased levels of recruiting chemokines [22,23]. Also, DCs frequencies have been reported to be increased in the BAL of IPF patients [23,24].

Given these observations, as to our knowledge no efforts have been focused on peripheral DCs, the aim of the present study was to investigate the frequency distribution of circulating blood DCs subsets in IPF patients at the time of first diagnosis and in response to currently available anti-fibrotic therapies. Assessment of peripheral reactive oxygen species (ROS), of serum expression levels of pro-inflammatory, and of Th-1 and Th-2 polarizing cytokines was performed as well. Correlations with lung function, disease severity indices and survival were analyzed.

2. Materials and methods

2.1. Study population

Sixty patients affected by IPF, referring to our Division between May 2014 and May 2018, were enrolled at the time of first observation. IPF diagnosis was made according to the 2011 official diagnostic criteria [25]. Patients with concomitant lung cancer or acute exacerbation at enrolment were excluded. Thirty age- and sex-comparable volunteers with no lung disease, including patients relatives or subjects referring to us for lung function screening, were enrolled as control group.The study was conducted in accordance with the amended Declaration of Helsinki. The local Ethics committee approved the study, data were collected in an anonymous way and all individuals gave written informed consent. All IPF patients underwent a complete lung function testing. Spirometry, lung volumes measurement and determination of the haemoglobin (Hb)-adjusted single-breath diffusing lung capacity of the carbon monoxide ($DLCO_{sb}$) were performed using a computer-assisted spirometer (Quark PFT 2008 Suite Version Cosmed Ltd. Rome Italy) according to international standards [26–28]. The 6-minute walk test (6-MWT) was performed by trained hospital staff according to guidelines [29]. The gender-age-physiology (GAP) and TORVAN disease staging indices were recorded as previously described [30]. Lung function screening of HCs included only basal spirometry and rest pulse-oximetry along with a detailed clinical history and physical examination.

2.2. Frequency distribution of circulating DC subsets

A DCs enumeration kit (Blood Dendritic Cell Enumeration Kit, Milteny Biotech, Bergisch Gladbach, Germany) was used to identify by flow cytometry the DC subtypes, according to the manufacturer's instructions. Reference median frequencies of blood DC subsets were 0.27 (range, 0.09–0.42), 0.02 (range, 0–0.04), 0.19 (range, 0.09–0.37) for cDC2, cDC1, and pDC, respectively. Briefly, 300 μL of EDTA-treated whole blood was incubated for 10 min with a cocktail of monoclonal antibodies (mAbs) including anti-CD1c (PE), anti-CD141(APC) and anti-CD303 (FITC). Mouse IgG2a-PE, IgG1-FITC, and IgG1-APC Abs were used as isotype controls. Samples were co-stained with CD19-PE-Cy-5 and CD14-PE-Cy5 to exclude B cells and monocytes from the analysis. Dead-cell discriminator was utilized in all specimens. After staining and erythrocyte lysis, cells were washed and fixed. Samples (5×10^5 events/sample) were acquired on a FACS CANTO II Flow Cytometer (Becton Dickinson Immunocytometry Systems, Palo Alto, CA, USA) and data analysis was performed using the FACS DIVA software (Becton Dickinson Immunocytometry Systems, Palo Alto, CA, USA). The gating strategy for the identification of each DC subtype is shown in Fig. 1.

Results were represented as percentage and absolute numbers of cDC2, cDC1 or pDC among total white blood cells (WBCs). Absolute numbers were calculated as follows: percent of a given DC subset x total number of white WBCs per $mm^3/100$. The WBCs count was determined using a Sysmex XT-1800i hemocytometer (Sysmex Europe, Norderstedt, Germany).

2.3. Oxidative burst analysis

OB was measured as ROS production by peripheral granulocytes by means of the PHAGOBURST™ assay (BD Biosciences La Jolla, CA, USA) according to the manufacturer's instructions. In particular, 100 μL of whole heparinized blood was incubated with the various stimuli at 37 °C for 10 min. A sample without stimulus served as negative background control. Non-fluorescent di-hydro-rhodamine (DHR) 123 was added to the samples for 10 min to allow the conversion to fluorescent rhodamine 123 upon ROS production by *E. coli* stimulated cells. The addition of a lysing solution for 20 min at room temperature removed erythrocytes. After one washing step, 200 μL of DNA staining solution was added for 10 min to exclude aggregation artifacts of bacteria or cells. Each experiment was performed in duplicate. For standardization purposes, for each sample 15.000 events were acquired within the gate of granulocytes using a FACS CANTO II flow cytometer. The measure of ROS production was calculated as mean fluorescence intensity (MFI) reflecting the enzymatic activity. The analysis was realized with the FACS DIVA software, according to the manufacturer's instructions.

2.4. Cytokines assays

Serum levels of tumor necrosis factor (TNF)-α, IL-6, interferon (INF)-γ, IL-2, IL-4, and IL-10 were respectively measured by the Cytometry bead array-Human Th-1/Th-2 cytokines (purchased from BD Biosciences). Each sample was analyzed in duplicate. Samples were acquired using a flow cytometer FACS CANTO II and analyzed with the FCAP array software (BD Biosciences, La Jolla, CA, USA), according to the manufacturer's instructions.

2.5. Statistics

Quantitative variables were characterized using mean ± standard deviation (SD) or median with quartile ranges, where appropriate, while categorical factors were described with absolute frequencies and percentages. Accordingly, the comparison between IPF patients and healthy controls was based on the Mann-Whitney *U* test. Where appropriate, comparisons were performed with the Wilcoxon test for paired data. Correlations among quantitative variables were based on the non-parametric Spearman rank correlation coefficient. Receiver operating characteristic (ROC) analysis was performed to identify the optimal cut-off value to group patients in those with low or high values. Survival was evaluated using Kaplan-Meier curves and compared by the log-rank test. The Overall Survival (OS) time was calculated from the date of diagnosis to the date of death or censored at the date of the last follow-up (FU) visit. The Cox proportional hazards model was used to calculate adjusted hazard ratios (HRs) and their 95% confidence interval (CI). All tests were two-tailed and statistical significance was set at $p < 0.05$. Variables with a p value < 0.05 in univariate analyses were tested in the multivariate analysis. Statistical analyses were performed using the GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA) or the MedCalc (MedCalcSoftware, Ostend, Belgium) platforms.

Table 1
Demographics and clinical features of the study population.

Parameter	Healthy subjects	IPF patients	p
Age (yr)	66 [61–71]	69 [66–72]	ns
Gender (M)	22 (73%)	47 (78%)	ns
BMI (Kg/m^2)	29 [25.6–30.8]	29.1 [25.8–31.2]	ns
Smoking status (current/former/never smokers)	8/12/10	0/41/16	ns
Pack/yr	34.00 ± 16.76	41.07 ± 21.11	ns
GAP stage (I/II/III)	na	16/23/21	–
TORVAN (1/2/3/4)	na	20/11/26/3	–

Data are expressed as absolute number (%), median [25th–75th] and mean ± SD, where appropriate.
Abbreviations: IPF = idiopathic pulmonary fibrosis; M = male; BMI = body mass index;
GAP = gender-age-physiology; na = not applicable; ns = not significant.

Table 2
Distribution of absolute numbers of peripheral DC subsets in the study population.

Parameter	Healthy subjects	IPF patients	p
cDC2 CD1c$^+$/mm^3	6.433 [3.89; 16.67]	4.54 [2.82; 6.03]	0.0043
cDC1 CD141$^+$/mm^3	0.813 [0.46; 1.73]	0.33 [0.11; 0.76]	0.0023
pDC CD303$^+$/mm^3	7.81 [4.42; 15.72]	2.8 [1.61; 4.78]	< 0.0001

Data are expressed as median [25th–75th].
Abbreviations: DC = dendritic cells; cDC = conventional DC; pDC = plasmacytoid DC; IPF = idiopathic pulmonary fibrosis.

Table 3
Lung function study in idiopathic IPF patients.

Parameters	
PaO_2 (mmHg) at rest in ambient air	75.5 [58–80]
SpO_2 at rest in ambient air	97.0 [93–98]
FVC (% predicted)	70.5 [57.5–91.2]
TLC (% predicted)	61.0 [51.0–76.0]
$DLCO_{sb}$ (% predicted)	47.0 [36.0–58.0]
6MWT (m)	374 [265–492.3]

Data are expressed as median [25th–75th].
Abbreviations: IPF = idiopathic pulmonary fibrosis; PaO_2 = arterial oxygen partial pressure;
SpO_2 = arterial oxygen saturation; FVC = forced vital capacity;
TLC = total lung capacity; $DLCO_{sb}$ = single-breath diffusing lung capacity of carbon monoxide;
6MWT = 6-minute walk test; m = meters.

Table 4
Serum levels of pro-inflammatory, Th-1 and Th2-polarazing cytokines in the study population.

Cytokine (pg/ml)	Healthy subjects	IPF patients	p
IL-6	3.45 [2.26–5.01]	7.02 [3.64–22.77]	0.0003
TNF-α	3.82 [1.10–1.38]	3.08 [1.38–4.26]	0.807
IFN-γ	2.09 [0.47–3.29]	1.73 [0.94–2.5]	0.521
IL-2	4.24 [2.29–5.32]	0.90 [0.47–1.28]	< 0.0001
IL-4	3.69 [0.89–4.68]	2.21 [1.36–3.00]	0.051
IL-10	2.58 [1.57–4.02]	2.42[1.73–3.27]	0.825

Data are expressed as median [25th–75th].
Abbreviations: IPF = idiopathic pulmonary fibrosis; Th = T helper; IPF = idiopathic pulmonary fibrosis; IL = interleukin; TNF = tumor necrosis factor; IFN = interferon.

3. Results

3.1. Patient characteristics

Our study population was composed of 60 clinically stable and therapy-naïve IPF patients and 30 sex- and age-matched healthy controls. Demographics and clinical data are shown in Table 1. Lung function parameters of IPF patients are reported in Table 2. After enrolment, 54 (90%) IPF patients initiated treatment with currently available anti-fibrotic drugs, including pirfenidone (n = 29) and nintedanib (n = 25). The remaining 6 patients were not eligible or refused treatment.

3.2. All peripheral dendritic cell subsets are significantly depleted in IPF patients

Flow cytometry estimates of frequencies of circulating DC subsets in our study cohort are reported in Fig. 1B. The relative median values [25th; 75th percentile] for the different DCs subsets were respectively 0.120 [0.07; 0.23] for CD1c + cCD2s (Fig. 1B, panel a), 0.014 [0.01; 0.03] for CD141$^+$cDC1s (Fig. 1B, panel b), and 0.140 [0.08; 0.23] for CD303$^+$pDCs (Fig. 1B, panel c) in healthy individuals. In the IPF group, the relative median percentages were 0.061 [0.03; 0.08] for CD1c$^+$ cDC2s (Fig. 1B, panel a), 0.004 [0.002; 0.01] for CD141$^+$cDC1s (Fig. 1B, panel b) and 0.039 [0.02; 0.06] for CD303$^+$pDCs (Fig. 1B, panel c). As reported, frequencies of all DC subsets were significantly lower in IPF patients as compared with the control group. Similarly, despite the WBCs count was higher in IPF than in HCs (7360/mm^3 [6205–8638] *vs* 6290/mm^3 [5260–7200], p 0.002), the absolute numbers of all DC subsets were significantly reduced in IPF patients as

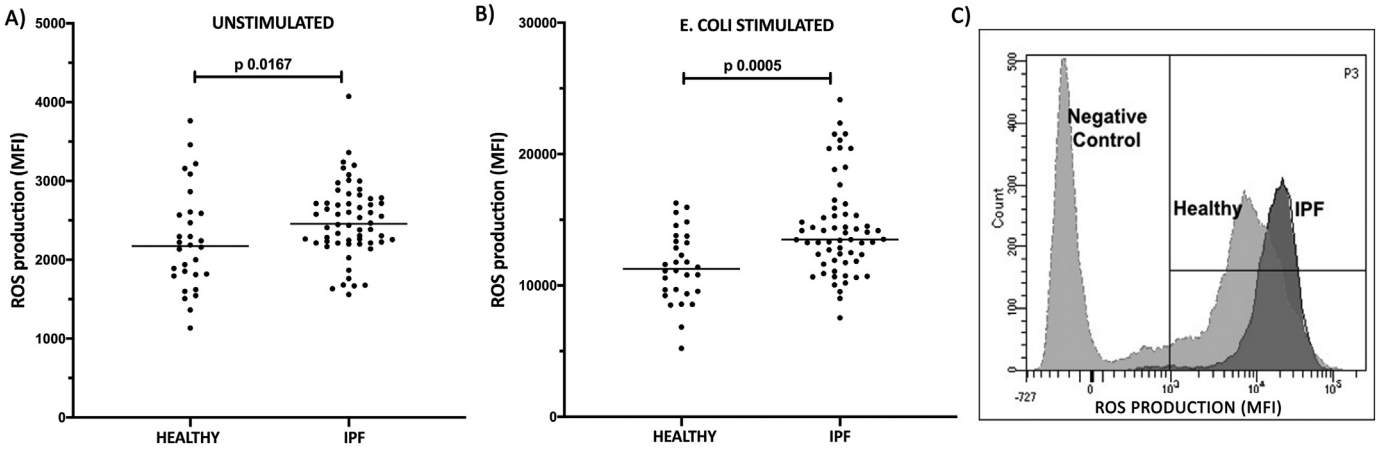

Fig. 2. Peripheral reactive oxygen species levels are higher in idiopathic pulmonary fibrosis patients than in healthy controls.
Scatter plots showing the distribution of reactive oxygen species (ROS) levels in unstimulated (A) and *E. coli* induced granulocytes (B) of healthy subjects and idiopathic pulmonary fibrosis (IPF) patients. C) Representative histograms of the *E.coli* induced ROS production in an healthy subject and in an IPF patient.

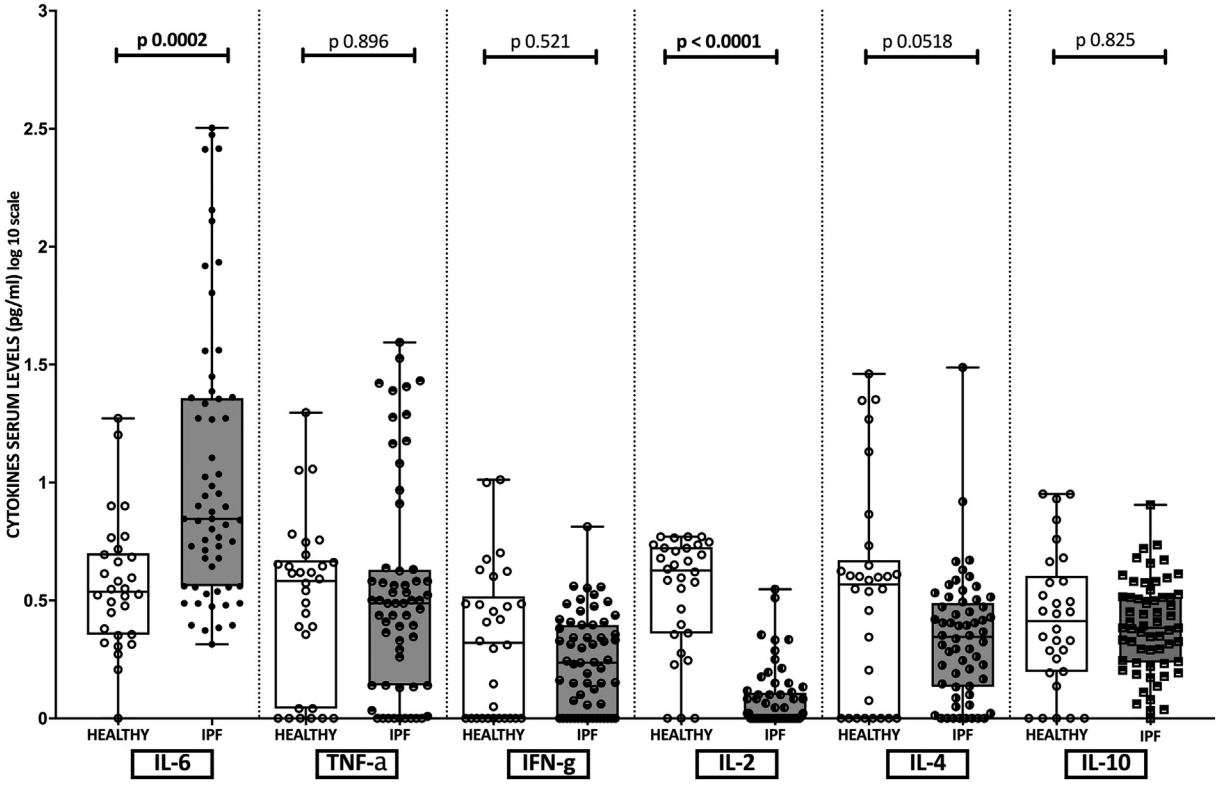

Fig. 3. Serum concentrations of interleukin-6 are increased in patients with idiopathic pulmonary fibrosis.
Scatter plots showing the distribution of the different cytokines analyzed in healthy subjects and idiopathic pulmonary fibrosis (IPF) patients.

compared to healthy controls, as analytically shown in Table 3.

Frequencies distribution of DC subsets was not correlated with smoking and lung function parameters. Also, no differences in the distribution of DCs were found through the stratification of IPF patients according to the GAP and TORVAN (grouping patients in TORVAN 1/2 *vs.* 3/4) disease staging scores.

3.3. Peripheral levels of reactive oxygen speciesare significantly increased in IPF patients

To investigate whether depletion of blood DC subsets was co-existing with an oxidative environment, peripheral levels of OB were estimated through the measurement of ROS release by blood granulocytes stimulated with *E. coli*. As illustrated in Fig. 2, both basal (panel A) and *E.coli* induced (panel B) ROS production was significantly increased in IPF patients with a median MFI value of 13,506 [11,761; 15,769] as compared to 11,264 [9333; 13,381] in the control group, in stimulated granulocytes. A fluorescence histogram representative of the distribution of *E.coli* induced ROS expression in an healthy subject and in an IPF patient is shown in Fig. 2C. ROS levels were not associated in IPF patients with smoking,lung function parameters, or with the frequency distribution of DCs. Also, no differences in ROS levels were found by stratification of IPF patients according to the GAP and TORVAN (grouping patients in TORVAN 1/2 *vs.* 3/4) disease staging scores.

3.4. Serum levels of the pro-inflammatory cytokine IL-6 are increased in IPF patients and correlate with disease severity and CDc1$^+$cDC2s frequencies

Serum levels of the pro-inflammatory cytokines IL-6 and TNF-α, of the Th-1 polarizing cytokines INF-γ and IL-2, and of the Th-2 polarizing cytokines IL-4 and IL-10 were measured to assess the IPF-related peripheral immune-inflammatory milieu. Overall quantitative data for each cytokine are reported in Table 4. IL-6 concentrations were significantly increased in IPF patients as compared to the control group, as also shown in Fig. 3, ranging from a minimum of 2.06 pg/ml to a maximum of 319.1 pg/ml. No differences were detected when looking at the other cytokines analyzed with the exception of IL-2 that resulted significantly reduced in the cohort of IPF patients (Fig. 3). Of note, the highest levels of IL-6 levels were measured in advanced IPF patients according to the GAP staging (Fig. 4). This finding was consistent with a significant negative correlation between IL-6 serum levels and two main lung function parameters, that are the FVC ($r = -0.29$, $p = 0.046$) and the $DLCO_{sb}$ ($r = -0.33$, $p = 0.028$) (both expressed as % predicted), along with the arterial oxygen partial pressure (mmHg) at rest in ambient air ($r = -0.35$, $p = 0.013$). No differences of IL-6 levels were observed in the case of patients stratification according to the TORVAN score (grouping patients in TORVAN 1/2 *vs.* 3/4). Correlation analysis between IL-6 serum levels and the frequency distribution of all DC subtypes further showed that in IPF patients the former was negatively related with the circulating CD1c$^+$cDC2 subpopulation ($r = -0.27$, $p = 0.046$). No significant correlations were found with the CD141$^+$cDC1 and CD303$^+$pDC subsets, with the peripheral levels of ROS, or with smoking.

3.5. Peripheral frequencies of DC subsets and levels of ROS and IL-6 are not modified by anti-fibrotic therapies

To address whether currently available anti-fibrotic drugs may affect the immune-inflammatory perturbations we observed in therapy naïve patients, the frequency distribution of peripheral DC subsets, along with the peripheral ROS and IL-6 levels, measured in a sub-cohort of 29 clinically stable patients after 12 months of treatment. Fourteen patients were receiving pirfenidone (2403 mg/day) and 15 nintedanib (300 mg/day). None of them experienced any acute exacerbation or severe drug-related adverse event requiring drug discontinuation during the study period. As shown in Fig. 5A, the frequency distribution of blood DC subsets was not modified upon treatment, with no differences related to the two drugs used. Also, IL-6 and ROS levels did not

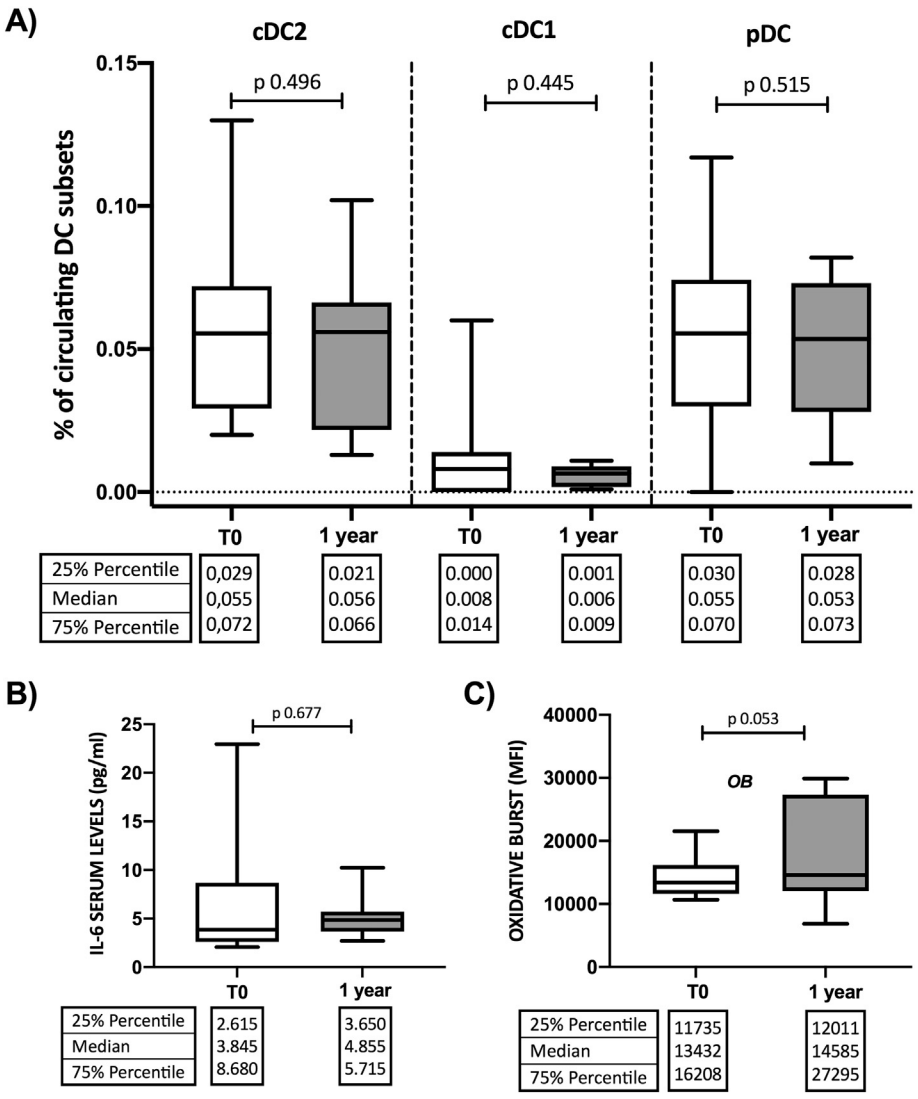

Fig. 4. Increased interleukin-6 serumlevels correlate with severity of idiopathic pulmonary fibrosis.

significantly differ in treated patients as compared with baseline values (Fig. 5B-C). Detailed quantitative data are reported for all measurements in the bottom of Fig. 5.

3.6. Low frequencies of peripheral cDC2 and high IL-6 levels are associated with reduced survival in IPF patients

To investigate the association between the frequencies of peripheral DC subsets, IL-6 and ROS levels and the OS, a log rank test was performed for the whole cohort of IPF patients. Lung function parameters, including the FVC and the $DLCO_{sb}$, were taken into account as well. Results showed that IPF patients with low frequencies of $CD1c^+$cDC2s (cut-off value of < 0.09%) had a significant worse survival ($p = 0.029$) (median survival 44.65 months; HR 3.35; 95% CI of ratio1.26–8.94) as compared with patients with higher $CD1c^+$cDC2 percentages (median survival > 55 months; HR 0.29; 95% CI of ratio 0.11–0.79) (Fig. 6A). In addition, we observed that IPF patients with higher IL-6 values (cut-off value of > 4.74 pg/ml) had a reduced survival ($p = 0.013$) (median survival 35.28 months; HR 3.49; 95% CI of ratio 1.29–9.45) with respect to patients with lower IL-6 values (median survival 53.21 months; HR 0.28; 95% CI of ratio 0.10–0.77) (Fig. 6B). No significant associations were found with $CD141^+$cDC1 and $CD303^+$pDC frequencies and with ROS production. Lastly, when looking at the association of OS with lung function, we found that IPF patients with FVC and $DLCO_{sb}$ measurements lower than their respective median values (< 76% and < 49% of predicted) had a worse prognosis with a median survival of 31.27 months (HR 5.64, 95% CI of ratio 2.00–15.90; $p = 0.001$) andof 33.80 months (HR 4.32; 95% CI of ratio 1.53–12.16; $p = 0.003$), respectively. Overall, data did not differ in sub-group analysis comparing patients treated with pirfenidone *vs.* nintedanib (not shown).

3.7. Peripheral $CD1c^+$cDC2 frequencies are predictive of survival in IPF patients

To assess the predictive value of demographic, clinical and biological parameters on the OS of our IPF study cohort, Cox regression analysis was performed including age, gender, smoking, body mass index (BMI), FVC, $DLCO_{sb}$, frequencies of cDCs (cDC2 andcDC1 subsets) and pDCs, ROS and IL-6 levels. Univariate analysis showed that FVC, $DLCO_{sb}$, $CD1c^+$cDC2 frequencies and IL-6 serum levels were all OS predictive factors in IPF patients, with detailed statistical data reported in Table 5A. In multivariate analysis the FVC and the frequency distribution of the $CDc1^+$cDC2 subset were the only two independent predictive factors of OS (Table 5B). Overall, no differences were reported in sub-group analysis comparing patients treated with pirfenidone *vs.*nintedanib (not shown).

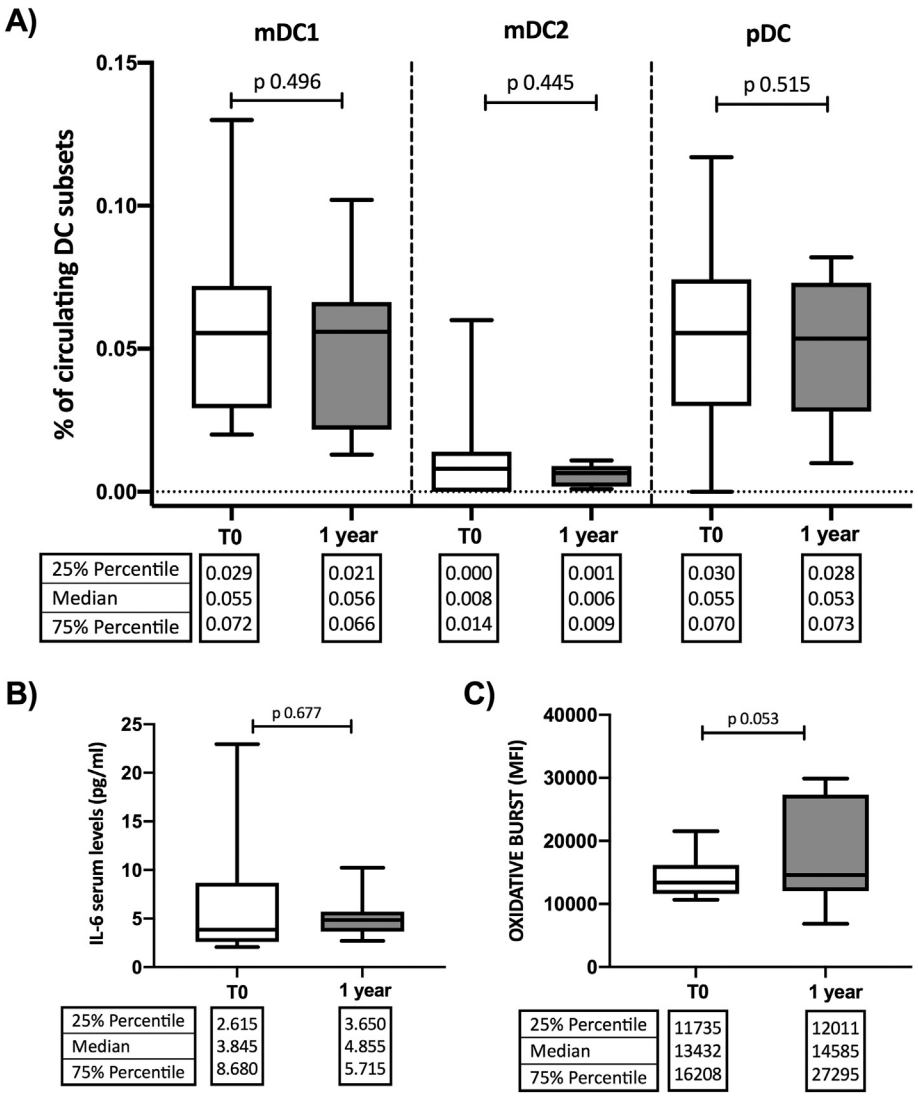

Fig. 5. Peripheral frequencies of DCs and interleukin-6 and reactive oxygen species levels are not modified by anti-fibrotic drugs.

4. Discussion

To the best of our knowledge, this is the first report addressing the frequency distribution of blood DC subsets in the clinical setting of IPF. Our results show that circulating conventional DCs (cDC2 and cDC1) and pDCs are markedly depleted in clinically stable and therapy naïve IPF patients at the time of first diagnosis. This finding is associated with an inflammatory milieu characterized by increased levels of peripheral ROS and IL-6 expression, and is not modified by currently available anti-fibrotic therapies. Also, baseline serum IL-6 production increases along with disease severity and is negatively correlated with the frequency of $CD1c^+$cDC2s. Very interestingly, both low $CD1c^+$cDC2s and high IL-6 levels are associated with a reduced survival, the former being an independent predictive factor of worse prognosis.

Notably, cumulative literature data have elucidated the key role that the different subsets of DCs play in the orchestration of the host immune response [31]. Also, there is growing evidence that either quantitative reduction or dysfunction of DCs are features shared by different diseases, including chronic obstructive lung disease and obstructive sleep apnea [32,33]. The involvement of DCs in the pathogenesis of IPF is a challenging field of quite recent interest, with only few reports available in humans. To date, immature DCs have been shown to infiltrate the lungs of IPF patients specifically in areas of epithelial hyperplasia and fibrosis. Conversely, mature DCs seem to accumulate in well organized, lymph node-like structures and in the BAL of IPF patients [24,34]. Altogether, these observations are of relevance as suggest a sort of compartmentalization of distinct functional DC subsets in the IPF lung.

Herein, we clearly demonstrate that the frequencies of circulating conventional and plasmacytoid DC subsets are significantly reduced in IPF patients. To further strength this observation we also show that depletion of DC subsets in IPF is confirmed when looking at their absolute numbers. Our data are quite significant if we take into account that enumeration and phenotypic analysis of DCs suffer from their low percentages at the peripheral level even in healthy individuals. Previous reports have shown that hyperplastic epithelial cells and fibroblasts located in the fibroblastic foci of IPF patients are able to recruit blood DCs through the expression of chemokines like CCL19, CXCL12, and CCL21 [22,35]. In particular, the ability of lung fibroblasts obtained from IPF patients to modulate the activation and maturation of DCs has been further confirmed in *in vitro* models of co-cultures [36]. In line with these findings, our belief is that the depletion of peripheral DCs we observed in our IPF cohort may be likely attributable to the tissue recruitment at the lung level, where they are involved in fibrogenesis, suggesting their increased turnover. Undoubtedly, this hypothesis has to be confirmed in future lung-blood comparative studies as any impairment at the bone marrow level cannot be excluded as well.

Peripheral depletion of all DC subsets was co-existing with increased

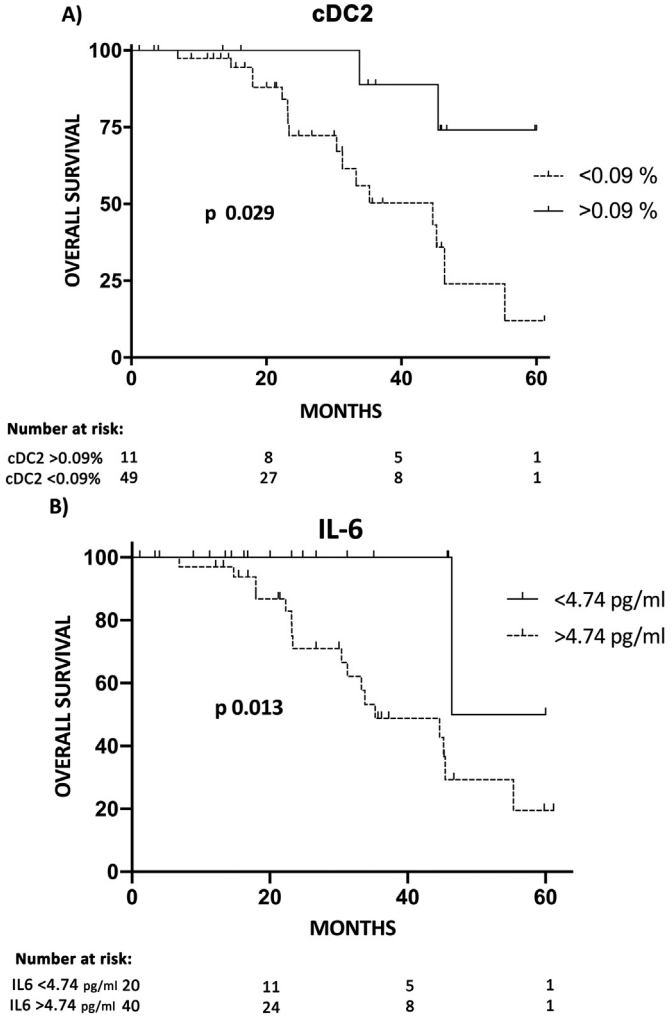

Fig. 6. Low frequencies of circulatingcDC1 and high interleukin-6 serumlevels are associated with reduced survival in idiopathic pulmonary fibrosis patients. Kaplan Meier curves representative of the overall survival (OS) of idiopathic pulmonary fibrosis (IPF) patients according to the baseline (at diagnosis) frequencies of circulating cDC2(panel A) and serum levels of interleukin (IL)-6 (panel B).

levels of OB, as assessed by the measurement of ROS release by *E. coli*-stimulated blood granulocytes. It is widely recognized that oxidative stress is a key piece of the IPF pathogenesis puzzle [8,37], and that ROS stimulate fibroblasts and alveolar epithelial cells to produce large amounts of IL-6 and IL-8 [38–40]. Of note, previous studies demonstrating the increased IL-6 serum levels in IPF patients, have already revealed the potential relationship between this pro-inflammatory cytokine and the development of fibrosis [41–43]. Accordingly, IL-6 neutralizing antibodies have been shown to reduce lung inflammation and fibrosis in a mouse model [41]. In line with these observations, our results confirm that IL-6 production is significantly increased in the sera of IPF patients, with median values doubling those of the control population of healthy subjects. Interestingly, we observed that IL-6 levels increase along with disease severity, as shown by the higher expression in IPF patients in GAP 3 stage, being negatively correlated with both the FVC and the $DLCO_{sb}$. This observation was not confirmed when patients were stratified according to the TORVAN staging score. Since we excluded patients with lung cancer at enrolment in order to reduce any bias with our results, in our opinion, such an observation further reinforces our data as they seem to be disease-specific as not influenced by co-morbidities. Most importantly, we also found that IL-6 expression is inversely related to the frequency distribution of peripheral $CD1c^+$cDC2s. It is known that IL-6 is a pivotal cytokine involved in the regulation of DC differentiation by affecting the transition from the resting/immature to the activated/mature phenotype. Such an effect results in a reduced ability of DCs to activate T cells, suggesting that IL-6 may act as an immune-suppressive factor in this context [44–46]. In particular, DCs dysfunction seems to be specifically mediated by the IL-6/STAT-3 signaling pathway which is also an emerging key player involved in pulmonary fibrosis [41,44,47–49]. Altogether, these findings are intriguing as they may be suggestive of a concomitant functional perturbation of these cells that certainly merits to be investigated further in the setting of IPF, with a special attention to $CD1c^+$cDC2s. This because $CD1c^+$cDC2s are the major subtype of blood DCs and act as potent inducers of naïve T cells. Also, as cDC2s are the main effectors in cancer immunity, any perturbation of these cells might account for the increased risk of cancer in IPF patients. The ongoing process of phenotypic and functional assessment of human DCs has recently allowed the identification of a sort of heterogeneity within the pool of CD1c-positive DCs thought the characterization of a new subset, named AXL^+ $SIGLEC6^+$ DCs (AS DCs). AS DCs can express either CD1c or CD123, being morphologically similar to cDCs while expressing pDC markers, with a strong ability to activate T cells [17]. Taking into account such a

Table 5
Clinical and immune-inflammatory features predictive of survival in IPF patients

Variable	HR	95% CI for HR	p
A) Univariate analysis			
Age (yr)	0.847	0.332 to 2.158	0.728
Gender (M *vs.*F)	0.897	0.331 to 2.426	0.830
BMI (Kg/m^2)	0.429	0.150 to 1.226	0.114
Smoking status (current/former *vs.* never smokers)	0.410	0.118 to 1.419	0.159
FVC (% predicted)	5.437	1.208 to 24.469	0.027
$DLCO_{sb}$ (% predicted)	7.938	1.747 to 36.058	0.007
% of circulating cDC2s	5.255	1.188 to 23.241	0.028
% of circulating cDC1s	1.405	0.516 to 3.827	0.505
% of circulating pDCs	0.756	0.245 to 2.334	0.627
IL-6 serum levels (pg/ml)	8.397	1.112 to 63.384	0.039
B) Multivariate analysis			
FVC (% predicted)	6.010	1.166 to 30.969	0.032
$DLCO_{sb}$ (% predicted)	2.333	0.460 to 11.849	0.305
% of circulating cDC2s	5.910	1.102 to 31.701	0.038
IL-6 serum levels (pg/ml)	5.061	0.621 to 41.206	0.129

Abbreviations: IPF = idiopathic pulmonary fibrosis; HR = hazard ratio; CI = confidence interval; M = male; F = female; BMI = body mass index; FVC = forced vital capacity; $DLCO_{sb}$; single-breath diffusing lung capacity of carbon monoxide; IL = interleukin; cDCs = conventional dendritic cells; pDCs = plasmacytoid dendritic cells.

revised taxonomy of DCs, future studies will enable a more complete understanding of the their involvement in the pathogenesis of IPF. The consequences of pDCs depletion should be addressed as well as they may help explain the association between viral infections and the increased risk of developing IPF [50].

An additional proof that further reinforces the link between IL-6 and CD1c$^+$cDC2s is the finding that alterations of both of them are significantly associated with a reduced overall survival in our IPF cohort. At the end of the study period (July 2019), mortality was 36.6%. Death was due to disease progression in 17 patients (86%), to acute exacerbation (13%) in 3, to lung cancer (9%) in 2, and to acute ischemic heart attack in one case (4%). Determination of IL-6 levels has been reported to be useful for diagnostic and prognostic purposes in other diseases, including cancer [51,52]. With respect to IPF, higher plasma levels of IL-6 have been found to characterize patients affected by acute exacerbations with worse prognosis without a clear association with the disease-specific survival [53]. Therefore, our finding focused on IL-6 adds a new brick to the current knowledge as our patient population was not including cases with acute exacerbation at enrolment. On the other hand, the impact of CD1c$^+$ cDC2s on disease behavior represents a completely new information with an even stronger clinical significance as they also independently act as a predictive factor of worse prognosis.

Lastly, to address whether the immune-inflammatory perturbations we observed in newly diagnosed IPF patients are influenced by currently available anti-fibrotic drugs, we measured the frequency distribution of circulating DCs and the ROS and IL-6 serum levels in a subcohort of 29 cases. We found that neither pirfenidone nor nintedanib are able to exert any modulating effect at one year from the initiation of treatment (the same results were obtained at 6 months, data not shown). In a similar way, anti-fibrotic therapies are not influencing the impact of IL-6 and CDc1$^+$ cDC2s on overall survival, at least in our cohort. As literature data are lacking in this scenario, we believe that our results are only of indicative value because of the relatively small sample size analyzed for each therapy arm. By this point of view, a limitation of our study is represented by the single center setting that necessarily requires the confirmation of our results in larger patient cohorts. Further, the lack of comparative data on lung derived samples is another issue that should be addressed along with the integration of functional assays.

5. Conclusions

To our knowledge this is the first report demonstrating that all circulating DC subsets are significantly depleted in IPF patients along with increased reactive oxygen species and IL-6 production. Also, this study identifies CD1c$^+$cDC2s and IL-6 as potential biomarkers whose perturbations are associated with a reduced overall survival, with CD1c$^+$cDC2s being an independent predictor of worse prognosis. Deciphering the involvement of the immune system in the pathogenesis of IPF has become a prosperous field of exploration only in the recent years. Our findings are promising as open new scenarios both in terms of better understanding of the biological mechanisms underlying the disease and of identification of easy to perform biomarkers predictive of clinical behavior.

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Author's contributions

DG and MB designed the research study, analyzed the data and wrote the manuscript; SZ performed the experiments and analyzed the data; AC, GP, and LC recruited patients and collected clinical data. All authors contributed to the manuscript writing and approved its final version.

Declaration of Competing Interest

All authors declare they have no conflicting interests for this study.

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