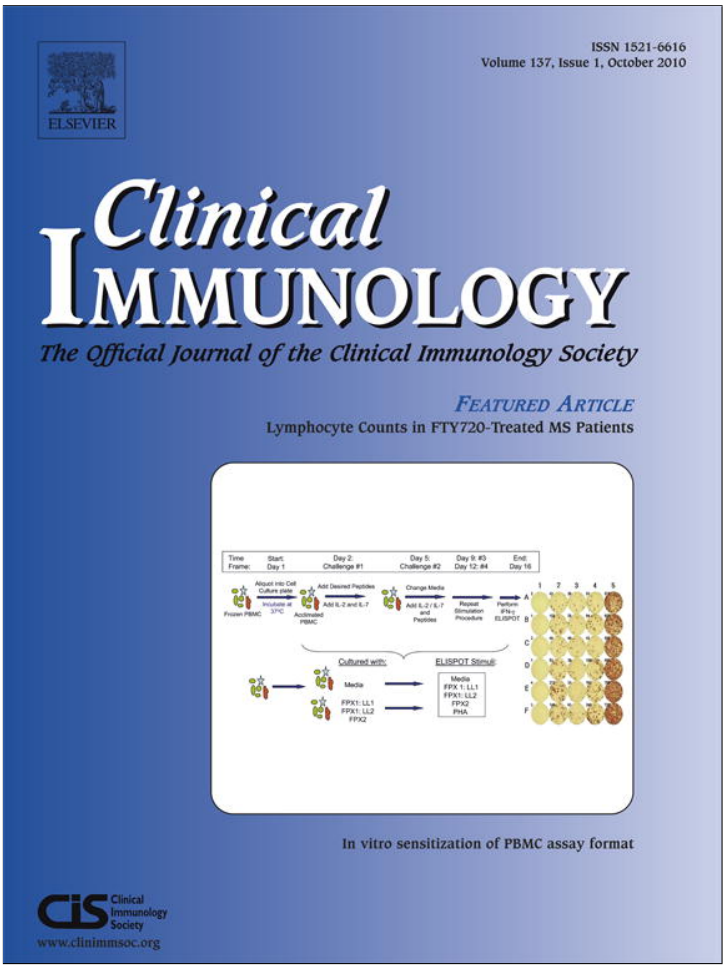

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Imbalance of circulating dendritic cell subsets in chronic obstructive pulmonary disease

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KEYWORDS
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Abstract Dendritic cells (DCs) play an unsettled role in chronic obstructive pulmonary disease (COPD) pathogenesis. Two main blood subsets, myeloid (m) and plasmacytoid (p) DCs, have been identified in humans. Phenotype and frequency of circulating DC subsets were assessed by multi-parametric flow cytometry in 28 COPD patients and 30 healthy controls (15 never smokers and 15 smokers). Proportion and absolute number of pDCs were significantly reduced in COPD patients in comparison with never smokers ($p<0.001$ and $p<0.003$) along with a marked increase of the mDC/pDC ratio ($p<0.001$). Analysis of peripheral lymphocyte subsets showed that the naive/memory T cell ratio was significantly reduced in COPD patients in comparison with never smokers ($p<0.001$). Similar perturbations in the distribution of DCs and T cells also occurred in control smokers. This study is the first report of an imbalance of blood DCs in COPD. Influence of smoking and clinical relevance of these findings are discussed.

Introduction

Chronic obstructive pulmonary disease (COPD) represents a significant cause of morbidity worldwide, being the fourth cause of death in most industrialized countries [1]. The disease is characterized by an ongoing inflammatory process and by continuous episodes of infection. Small airways remodeling and destruction of distal air spaces represent

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the main pathological abnormalities that lead to progressive/irreversible airflow limitation [2].

Cigarette smoke has been firmly established as the main risk factor for COPD. However, less than 20% of smokers develop a clinically significant disease, indicating the additional contribution of genetic and environmental factors [3]. Neutrophils, macrophages, and $CD8^+$ T lymphocytes are effector cells that participate in the inflammatory process of COPD, suggesting a cooperation of innate and adaptive immunity in the disease pathogenesis [4,5]. In addition, the persistence of airway inflammation after smoking cessation indicates an involvement of adaptive immune responses in COPD [6].

Dendritic cells (DCs) comprise heterogeneous subsets of bone-marrow-derived professional antigen presenting cells (APCs) [7]. DCs promote the activation of naive and memory T cells and orchestrate processes for the polarization of immune responses. In humans, two main circulating subsets, namely myeloid (m) and plasmacytoid (p) DCs, have been described according to origin, phenotype, and function [8,9]. To date, myeloid DCs comprise two subtypes: type-1 mDCs, which are regarded as the main APCs driving cell-mediated immune responses [7], and type-2 mDCs, which are still functionally poorly characterized. The other subset of DCs, which are pDCs, exerts potent antiviral activity due to the ability to produce type-I interferons (IFNs) [10,11] and is also thought to be critically involved in immune tolerance through a cooperation with regulatory T cells and in antitumor responses through their synergistic interaction with mDCs [12,13].

The role of DCs in the pathogenesis COPD is somehow unappreciated [14]. Data from a mouse model of COPD have shown an increased number of lung DCs in response to smoke [15]. Other animal and *in vitro* studies on the link between smoking, DCs, and COPD have led to contrasting results [16–20], and data in humans are relatively scarce [21–26]. To date, no significant differences have been reported in the number of immature $CD1a^+$ DCs in the large airways of COPD patients as compared with healthy smokers [21]. Conversely, there is evidence that immature DCs accumulate in the sputum, small airways, and alveoli of COPD patients suggesting a differential compartment location for distinct DC subtypes [22,23,26]. However, to our knowledge no study has so far analyzed the different DC subsets in COPD.

Here we enumerated and characterized the phenotype of circulating DCs in COPD patients using multi-parametric flow cytometry and assessed in parallel the contribution of cigarette smoke on DCs homeostasis in control groups. Differential analysis of peripheral T cell subsets was also performed.

Materials and methods

Study population

A total of 28 consecutive Italian patients affected by stable COPD who attended the outpatient service of the Respiratory Medicine Division of the Federico II University at the Monaldi Hospital in Naples, Italy, were enrolled in the study. Diagnostic preliminaries included physical examination, routine blood tests, lung function testing, and arterial blood gases analysis. Exclusion criteria were acute disease exacerbation (including viral and bacterial infections) and systemic administration of corticosteroids at enrolment or in the previous 60 days. Comorbidities included ischemic heart disease ($n=9$, 32.1%), systemic hypertension ($n=6$, 21.4%), and diabetes in 3 cases (10.7%). Thirty healthy age- and sex-matched volunteers were recruited as controls (15 never smokers and 15 current smokers). The local Ethics Committee approved the study and all patients provided written informed consent. Demographics and clinical characteristics of the study population are shown in Table 1.

Pulmonary function tests

Spirometry, determination of hemoglobin (Hb)-adjusted-single-breath diffusing capacity of the carbon monoxide ($TLCO_{SB}$), and measurement of CO uptake for alveolar volume (TLCO/VA) were

Table 1 Demographics and clinical characteristics of the study population.

Parameter	CTR NS [a] ($n=15$)	CTR SM [a] ($n=15$)	COPD [b] ($n=28$)
Age (years)	60.8 ± 3	60.1 ± 2.2	62 ± 4.8
M/F	11/4	11/4	19/6
Current smokers	–	15	9
Smoking history, pack/years	–	42.4 ± 18.6	46.9 ± 23
GOLD staging (*n* of patients)	–	–	I (0); II (16); III (6); IV (6)
FEV_1 (% of predicted)	101 ± 13	96.1 ± 12.1	51.2 ± 13.1 *
FEV_1/FVC (% of predicted)	82 ± 10	85.8 ± 9.5	59 ± 7.5 *
FEF_{25-75} (% of predicted)	92 ± 13	76.9 ± 19.3	20.5 ± 6.3 *
$TLCO_{sb}$ (% of predicted)	–	–	42.4 ± 17.6
TLCO/VA (% of predicted)	–	–	54.2 ± 24.5
PaO_2 (mmHg) at rest (21% FiO_2)	–	–	62 ± 9.6
N exacerbations/last 24 months	–	–	4 ± 1.4

Data are expressed as mean value ± SD.

[a] NS: never smokers; SM: smokers.

[b] Twelve patients (39.2%) were under treatment with inhaled bronchodilators (including long-acting beta-2 agonists and/or anticholinergic drugs), while the remaining 17 cases (60.8%) were on combination therapy with inhaled bronchodilators and corticosteroids. Twelve patients (42.8%) were under continuous long-term oxygen therapy.

* $p<0.01$ vs. CTR NS and vs. CTR SM.

performed using a computer-assisted spirometer (Master Screen Diffusion, Jaeger, Wuerzburg, Germany) according to the American Thoracic/European Respiratory Societies acceptability and reproducibility standards [27,28]. Spirometry was realized at baseline (inhalation therapy wash-out of 12 h) and after the administration of 400 μg of salbutamol in agreement with the Global Initiative for Chronic Obstructive Lung Disease diagnosis and management criteria [29]. Airflow limitation was clinically confirmed as evidence of a ratio of FEV_1 to FVC of less than 0.70 after bronchodilation.

Phenotypic analysis and enumeration of blood DC subsets

Flow cytometry identification of DC subsets was performed using a circulating DC enumeration kit (Blood Dendritic Cell Enumeration kit, Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Venous blood was drawn into 0.2% EDTA-containing Vacutainer tubes and aliquots (300 μl) were labeled for 10 min with a cocktail of specific monoclonal antibodies (mAbs) including anti-CD1c-1-PE (BDCA-1, type-1 mDC marker), anti-CD303-fluorescein isothiocyanate (FITC; BDCA-2, pDC marker), and anti-CD141-allophycocianin (APC; BDCA-3, type-2 mDC marker) or mouse IgG_{2a}-PE, IgG_1-FITC, and IgG_1-APC antibodies as isotype controls. Samples were co-stained with CD19-PE-Cy-5 and CD14-PE-Cy5, to exclude B cells and monocytes and a dead-cell discriminator reagent was included in all samples. Cells were then treated with lysis buffer to remove red cells, washed, and fixed. Samples (1×10^6 events/sample) were acquired on a flow cytometer (Cyan, DAKO Cytomation, Colorado, USA) and data analysis was performed using the Summit software (version 4.3, DAKO). Results were expressed as percentage of mDCs or pDCs among total cells. The absolute number per mm^3 of any DC subset was calculated as follows: percent of a given DC subset × total number of white blood cells (WBCs) per mm^3/100. The mDC/pDC ratio was calculated as follows: absolute number of type-1 plus type-2 mDCs/absolute number of pDCs. An aliquot of blood was used for WBC/mm^3 determination via hemocytometer (XT-1800i Sysmex Europe, Norderstedt, Germany).

Flow cytometric analysis of peripheral immune cell subsets

One venous blood aliquot was reserved for immune cell subset evaluation. Selection of lymphocytes was based on side/forward scatter. Lymphocyte subsets were identified in the leukocytes gate (side scatter versus CD45-FITC) by using the following mAbs: CD3 (FITC or Peridinin Chlorophyll Protein Complex, PerCP) for total T lymphocytes; CD4 (PE or PerCP) and CD8 (PerCP) for T cell subsets, CD19 (PE) for B lymphocytes and CD16/CD56 (PE) for natural killer (NK) cells. Detection of naive and mature T cells was assessed by expression of CD45RA (PE) and CD45RO (PE), respectively, while staining for CD28 (PE), CD25 (PE), and DR (PE) was performed to identify activated T lymphocytes. Matched isotype Abs were used as negative controls. All reagents were purchased from Becton Dickinson (San Diego, CA, USA).

Statistical analysis

The Kolmogorov–Smirnov test was used to confirm normality distribution assumption for both primary (mDC/pDC ratio) and secondary outcome variables. Mean differences among groups were assessed using the *F* ANOVA or the Welch corrected ANOVA where appropriate. Accordingly, *post-hoc* tests were based either on unpaired *t* test or on the Welch corrected *t* test. Bonferroni adjustment was used for pairwise comparisons. The Pearson correlation coefficient and Spearman rank test were used for correlation analysis. All tests were two-tailed and values of $p<0.05$ were considered significant. Confidence intervals were based on a confidence level of 0.95.

To measure the effective power of the statistical procedure, a post-experimental power analysis on the primary outcome was performed. The power curve was evaluated by using the upper bound of the confidence intervals for the standard deviation of the mDC/pDC ratio in the study groups. Due to the lack of any closed formula for the "alternative" distribution of the *F* Welch statistic, a simulation approach has been implemented. With the available sample size, a significance level of 0.05, and the hypothesized standard deviation pattern, the simulation revealed an 89.5% power of correctly rejecting the null hypothesis of no difference among groups when the true population means of COPD patients, never smoker, and smoker controls were, respectively, 3.5, 1.5, and 3.5. Statistical analysis was performed using R 2.9.1.

Results

Circulating type-1 mDCs and pDCs are reduced in COPD

Flow cytometry estimates of frequencies of circulating DC subsets were performed in COPD patients, healthy never smokers, and current smokers without COPD. Representative plots for DC subset analysis in a healthy never smoker control and in a COPD patient are shown in Fig. 1. As shown in Fig. 2A, the mean percentage of type-1 mDCs was significantly lower in COPD patients (mean ± SD: 0.44 ± 0.24; SEM: 0.04; 95% CI: 0.35–0.53; range: 0.07–0.88) as compared with never smoker controls (mean ± SD: 0.72 ± 0.19; SEM: 0.05; 95% CI: 0.62–0.83; range: 0.36–1.06; $p=0.003$). A slight reduction of type-1 mDCs was also detected in control smokers (mean ± SD: 0.6 ± 0.3; SEM: 0.08; 95% CI: 0.43–0.77; range: 0.15–1). The distribution of type-2 mDCs was not significantly different among the three study groups (Fig. 2B). The mean percentage of pDCs was significantly reduced in COPD patients (mean ± SD: 0.17 ± 0.15; SEM: 0.03; 95% CI: 0.12–0.23; range: 0.01–0.53) in comparison with never smoker controls (mean ± SD: 0.48 ± 0.13; SEM: 0.03; 95% CI: 0.40–0.55; range: 0.3–0.74; $p<0.001$), the proportion of pDCs being below 0.2% in the majority of cases (61.7%; Fig. 2C). Frequencies of pDC were widely distributed in healthy smokers, reaching very low percentages in some instances (mean ± SD: 0.29 ± 0.23; SEM: 0.06; 95% CI: 0.16–0.41; range: 0.02–0.71).

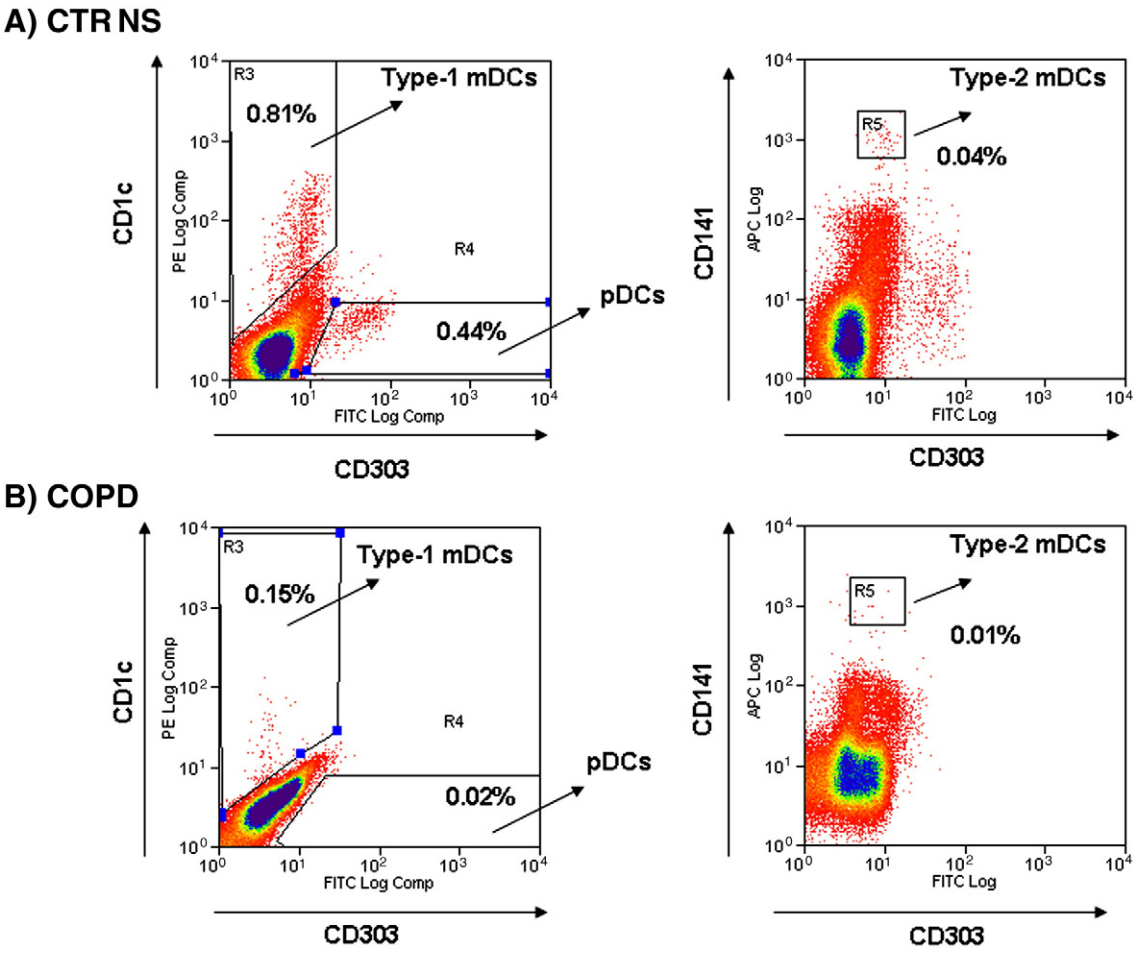

Figure 1 Representative flow cytometry analysis of type-1 mDC, type-2 mDC, and pDC subsets (A) in a healthy never smoker control (CTR NS) and (B) in a COPD patient. Numbers in the dot plots indicate the percentages of DC subsets among total blood cells after exclusion of dead cells, monocytes, and B lymphocytes.

Absolute numbers of blood pDCs are decreased in COPD

There were no differences in absolute numbers of type-1 mDCs between COPD patients (mean ± SD: 36.1 ± 22.3; SEM: 4.2; 95% CI: 27.5–45; range: 5.5–110), never smoker controls (mean ± SD: 46 ± 23; SEM: 5.8; 95% CI: 33.2–58.3; range: 16.1–99.3), and smokers (mean ± SD: 54 ± 28; SEM: 7.2; 95% CI: 38.4–69.5; range: 13–95.4; Fig. 3A). No differences in absolute numbers of type-2 mDCs were recorded (Fig. 3B).

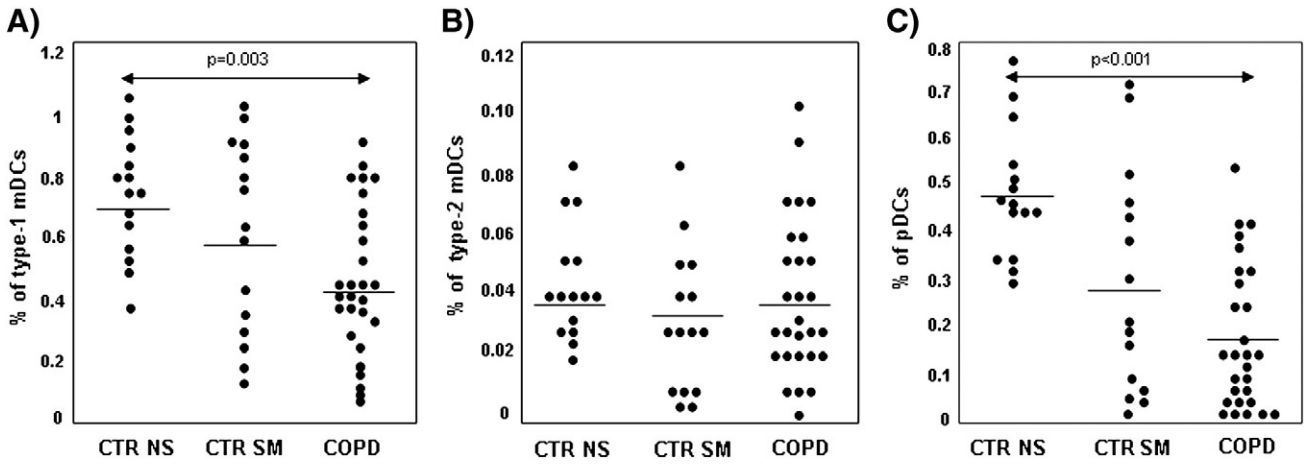

Figure 2 Percentages of (A) type-1 mDC, (B) type-2 mDC, and (C) pDC subsets among total blood cells in COPD patients, in healthy never smoker controls (CTR NS), and in current smokers (CTR SM) without COPD. Horizontal bars represent mean values.

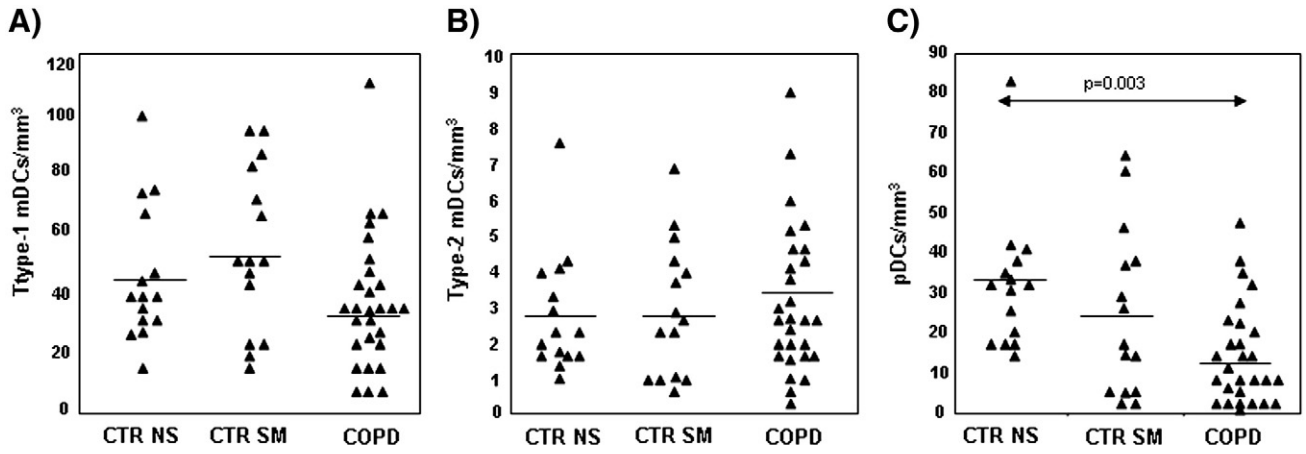

Figure 3 Absolute numbers of (A) type-1 mDC, (B) type-2 mDC, and (C) pDC subsets among peripheral leukocytes in COPD patients, in healthy never smoker controls (CTR NS), and in control current smokers (CTR SM) without COPD. Horizontal bars represent mean values.

Conversely, the mean absolute number of pDCs was significantly decreased in COPD patients (mean ± SD: 14.2 ± 12.3; SEM: 2.3; 95% CI: 9.4–19; range: 0.7–47.6) in comparison with never smoker controls (mean ± SD: 32 ± 17; SEM: 4.3; 95% CI: 22.7–41.3; range: 14–83; $p<0.0003$; Fig. 3C). The absolute numbers of pDCs were widely distributed in smokers (mean ± SD: 25 ± 21; SEM: 5.3; 95% CI: 13.1–36) ranging from very low values (2 cells/mm^3) up to 64 cells/mm^3.

DC imbalance in COPD

As shown in Fig. 4, the mDC/pDC ratio was significantly increased in COPD patients (mean ± SD: 4.2 ± 2.7; SEM: 0.5; 95% CI: 3.1–5.2; range: 1–10) in comparison with never smokers (mean ± SD: 1.7 ± 0.5; SEM: 0.1; 95% CI: 1.4–2; range: 0.9–2.5; $p<0.001$). An increase of this ratio was also found in control smokers (mean ± SD: 3.8 ± 2.5; SEM: 0.6; 95% CI: 2.5–5.2; range: 0.8–8.5).

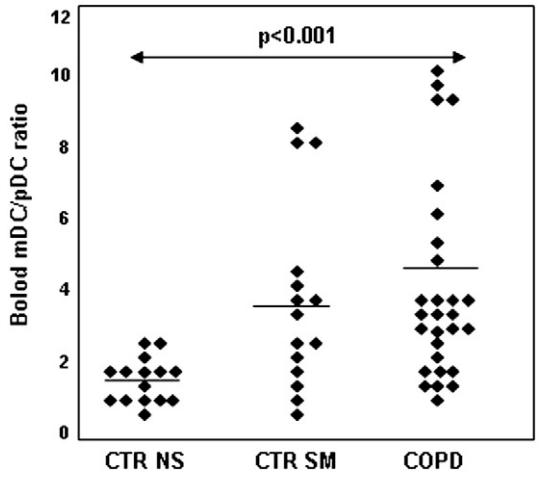

Figure 4 Distribution of the blood mDC/pDC ratio in COPD patients, in healthy never smoker controls (CTR NS), and in control current smokers (CTR SM) without COPD. Horizontal bars represent mean values.

Expansion of circulating mature T lymphocytes in COPD

Analysis of the peripheral lymphocyte compartment showed the lack of significant differences among the three study groups (Table 2). No differences in the proportion of T CD4$^+$, T CD8$^+$, B, natural killer (NK), and NKT cells were observed. Similarly, no differences in the proportion of CD3$^+$ T cells expressing the activation markers DR, CD28, and CD25 were recorded in the three study groups. However, COPD patients displayed a significant reduction in the proportion of naive CD3$^+$ T cells expressing CD45RA in comparison with never smokers ($p<0.01$). Conversely, the mean percentage of mature CD3$^+$CD45RO$^+$ T lymphocytes was significantly increased in COPD patients as compared with never smokers, along with a significant inversion of the naive/mature ratio ($p<0.01$). An inversion of the naive/mature ratio along with an increase of mature T cells also occurred in healthy smokers ($p<0.001$). Distribution of naive and mature CD3$^+$ T cells along with the blood naive/mature ratio in the three study groups is shown in Fig. 5.

DC and naive/memory T cell perturbations do not associate with COPD severity and smoking

No correlations were found between DCs (either as percentage or absolute numbers) or mDC/pDC ratio and COPD associated parameters of disease severity including lung function, therapy, smoking history, and number of exacerbations over the last 2 years. Finally, perturbations of naive/mature T cells did not correlate with DCs or COPD severity or smoking history. Similarly, perturbations of DC subsets and of naive/mature T cells were not correlated with smoking history in smokers without COPD.

Table 2 Phenotypic analysis of peripheral lymphocyte subsets.

Parameter	CTR NS (n=15)	CTR SM (n=15)	COPD (n=28)	p
Total WBCs/mm^3^	5137±1739	8567±1273	8015±1384	COPD vs. CTR NS, $p<0.001$ CTR SM vs. CTR NS, $p<0.001$
Total lymphocytes/mm^3	2155±576	2024±547	1879±577	ns*
% CD3$^+$ cells	73.1±5.9	74.4±8.4	71.1 ±12	ns
% CD3$^+$CD4$^+$ cells	44.4±7.5	45±9.8	40±10.8	ns
% CD3$^+$CD8$^+$ cells	25.4±4.6	25.3±9	29.4±13	ns
% CD3$^-$CD16$^+$/CD56$^+$ cells	11.6±4.7	12.2±6.4	16±11	ns
%CD3$^+$CD16$^+$/CD56$^+$ cells	5.4±4.3	4.1±3.6	6±4.4	ns
% CD19$^+$ cells	12±3.2	11±3.2	10±5.5	ns
CD3$^+$CD4$^+$/CD3$^+$CD8$^+$	1.8±0.5	2.1±1.1	1.6±0.8	ns
% CD3$^+$CD4$^+$CD8$^+$ cells	1.3±1.2	1.4±1.3	1.3±0.9	ns
% CD3$^+$CD45RA$^+$ cells	40.7±5.9	32.8±15.2	27.8±10.4	COPD vs. CTR NS, $p<0.01$
% CD3$^+$CD45RO$^+$ cells	32.3±7.2	41.6±12.5	42.3±8.4	COPD vs. CTR NS, $p<0.01$
CD3$^+$CD45RA$^+$/CD3$^+$CD45RO$^+$	1.3±0.5	0.9±0.8	0.6±0.3	COPD vs. CTR NS $p<0.001$
% CD3$^+$CD4$^+$CD28$^+$ cells	40.4±6.7	43.1±9.8	35±9.2	ns
% CD3$^+$CD4$^+$DR$^+$ cells	1±0.6	1.2±0.6	0.8±0.5	ns
% CD3$^+$CD4$^+$CD25$^+$ cells	1.3±0.5	1.2±0.4	1.5±0.6	ns
% CD3$^+$CD8$^+$DR$^+$ cells	0.7±0.5	0.8±0.4	0.5±0.5	ns

Data are expressed as mean value ± SD; ^range of normal values: 4000–11,000/mm^3; *ns: not significant.
All values are expressed as % of total lymphocytes.

Discussion

Although evident that cigarette smoke, the primary cause of COPD, significantly influences dendritic cell functions, little is known about the roles of DCs in the pathogenesis of COPD [30]. We show for the first time that frequency and absolute number of circulating pDCs are significantly reduced in patients with stable COPD. This finding associates with a consistent increase in peripheral mDC/pDC ratio, suggesting a perturbation of the balance between functionally different DC types in COPD. These findings did not correlate with parameters of COPD severity, smoking history, or treatment. Interestingly, although smoke is probably a pre-existing cause of this perturbation, and other factors are involved, we found that a subset of smokers without COPD had a significant depletion of pDCs. Overall, healthy smokers also displayed a trend toward an increase in the mDC/pDC ratio similarly to COPD patients suggesting a link between smoke and DC homeostasis that, however, requires further investigation. In this issue, selective screening of a larger number of healthy smokers (the only ones included in the present study) in comparison with smokers with chronic bronchitis without airflow limitation (previously referred as GOLD stage 0) would be helpful to address whether DC imbalance is associated with an increased risk for COPD development.

In agreement with previous observations [31,32], we found no significant perturbations in the distribution of circulating lymphocyte subsets in the three study groups. Conversely, the inversion of the naive/mature T cell ratio found in COPD patients was not unexpected and likely representative of a consequence of the natural history of the disease, which is characterized by recurrent episodes of viral and bacterial infections. In this instance, the paradigm that excessive antigenic stimulation accelerates T cell maturation is widely accepted in chronic inflammation. However, mature T cells were also increased in smokers without COPD likely due to their higher susceptibility to infection. This finding is quite surprising and likely suggests a non-peptide-mediated mechanism as cigarette smoke components may affect the integrity of the mucosa barrier at the bronchial level thus leading to an increased antigen exposure.

Our approach tried to minimize bias by enumerating and characterizing DCs by means of a highly performing multiparametric assay that allowed the discrimination of viable DCs without the need of preliminary cell separation, in a fashion similar to that reported by Hashizume et al. in atopic dermatitis patients [33]. Our reported imbalance of DCs in COPD follows reports of alterations in the distribution of DC subsets in human immunodeficiency virus infection, chronic viral hepatitis, psoriatic arthritis, and rheumatoid arthritis [34–37]. While it is known that cigarette smoke may influence *per se* the phenotype and function of DCs since low doses of nicotine induce DC maturation [19] and higher doses lead to opposite effects [17], studies in animals have often provided conflicting results. It has been shown that cigarette smoke impairs DC maturation in mice [16] whereas smoke has also been found capable to induce activation of DCs that can contribute to chronic airway inflammation through the release of DC-derived chemotactic factors [17]. In this context, airway inflammation and emphysema do not develop in CCR6-deficient mice due to a reduced accumulation of pulmonary DCs [18]. Further observations suggest cigarette smoke-primed DCs to strongly affect the recruitment of neutrophils and potentiate the proliferation of CD8 T cells [20,38].

In humans, the total number of bronchial DCs is reduced in COPD smokers in comparison with ex-smokers [25]. In addition, more recently it has been shown that myeloid DC differentiation is altered in small airways of current smokers and COPD patients in comparison with never smokers [39]. Conversely, Vassallo et al. have found that COPD is associated with increased numbers of cells bearing markers

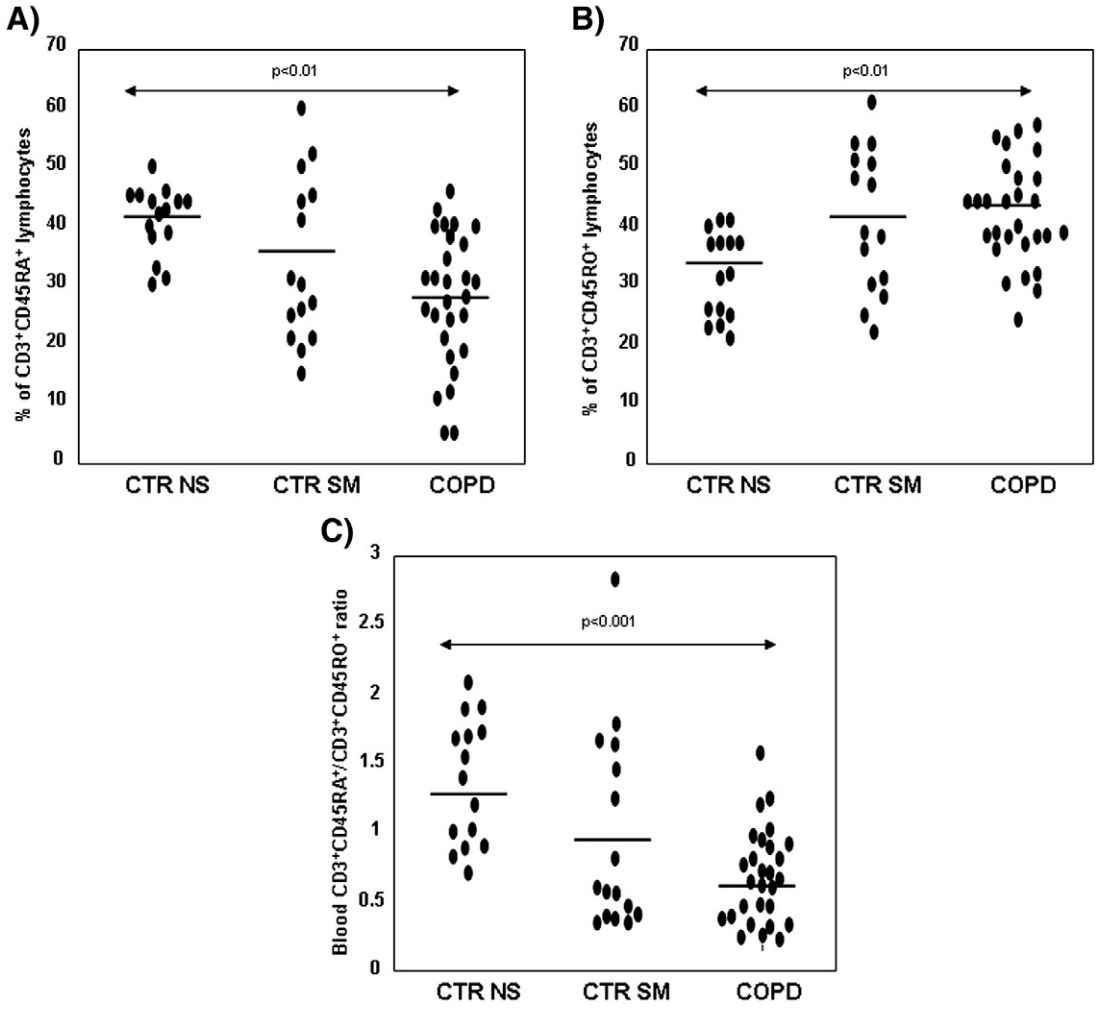

Figure 5 Distribution of the proportion of (A) circulating naive and (B) mature $CD3^+$ T lymphocytes in control non-smokers (CTR NS), control current smokers (CTR SM), and COPD patients. (C) Distribution of the blood ratio of $CD3^+CD45RA^+/CD3^+CD45RO^+$ lymphocytes in the three study groups. Horizontal bars represent mean values.

associated with Langerhans cells and mature mDCs, and that cigarette smoke promotes survival signals and augments survival of dendritic cells [40].

Although speculative, some interpretations of the data on mDCs and pDCs can also be done, particularly in view of reports of distinct immune modulatory functions of these cell subsets when present at different anatomical locations in the human lung [41,42]. As the majority of COPD patients displayed an "abnormal" mDC/pDC ratio, it cannot be excluded that the findings we described may be secondary to COPD. At least theoretically, any impairment/depletion of pDCs in the lung/draining lymph node levels could be hypothesized as influential on several alterations described in COPD [43], such as (a) persistence of inflammation (due to enhanced mDC activities); (b) increased susceptibility to viral infections; and (c) decreased immune tolerance.

In conclusion, the cause leading to the peripheral depletion of pDCs also needs to be identified since the selective lung recruitment of pDCs or, alternatively, an impairment of their generation at the medulla level cannot be excluded. A recent report by Van Pottelberge et al. suggests a GOLD stage-dependent accumulation of pDC in lymphoid follicles of COPD patients along with an enhanced production of TNF-alpha and IL-8 by maturing pDC [44]. In any case, longitudinal studies that will monitor changes of circulating pDC are required to address whether healthy smokers with low pDCs levels are at risk to develop COPD and whether DC imbalance may affect COPD progression.

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