Aberrant characteristics of peripheral blood innate lymphoid cells in COPD, independent of smoking history

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ILC1 and ILC2 populations in peripheral blood have different inflammatory profiles in COPD and asthma patients, which may have implications for patient stratification and therapy development
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Abstract

Background Distinguishing asthma and COPD can pose challenges in clinical practice. Increased group 1 innate lymphoid cells (ILC1s) have been found in the lungs and peripheral blood of COPD patients, while asthma is associated with elevated levels of ILC2s. However, it is unclear whether the inflammatory characteristics of ILC1s and ILC2s differ between COPD and asthma. This study aims to compare peripheral blood ILC subsets and their expression of inflammatory markers in COPD patients, asthma patients and controls.

Methods The study utilised multi-colour flow cytometry to analyse peripheral blood ILC populations in clinically stable COPD patients (n=38), asthma patients (n=37), and smoking (n=19) and non-smoking (n=16) controls.

Results Proportions of peripheral blood inflammatory $CD4^+$ ILC1s were significantly higher in COPD patients than in asthma. Proportions of $CD4^-$ ILC1s were increased in COPD patients compared to asthma patients and smoking controls. Frequencies of $CD117^-$ ILC2s were significantly reduced in COPD patients compared with asthma patients. In contrast, the fraction of inflammatory $CD45RO^+$ cells within the $CD117^-$ ILC2 population was significantly increased. Principal component analyses showed that combined features of the circulating ILC compartment separated COPD patients from asthma patients and both control groups.

Conclusion Our in-depth characterisation of ILC1 and ILC2 populations in peripheral blood revealed significant differences in their phenotypes between COPD and asthma patients and smoking or non-smoking controls. These findings suggest a role for both ILC subsets in COPD disease pathology, independent of smoking history, and may have implications for patient stratification and therapy development.

Introduction

COPD is a heterogeneous progressive lung disease with persistent airflow obstruction and respiratory symptoms, mostly caused by tobacco smoking or pollution exposure [1]. Because not all smokers develop COPD, it is thought that the aetiology of COPD involves a combination of cellular damage by inhaled particulate matter and genetic, early-life risk and social factors. Pathological alterations in COPD include epithelial cell reprogramming in combination with immune and tissue remodelling responses, leading to the development of emphysema [2, 3].

Recent evidence supports a role for natural killer (NK) cells and innate lymphoid cells (ILCs) in the pathogenesis of COPD, although the mechanisms involved are still elusive [4, 5]. The NK cell population is increased in the lungs of COPD patients in comparison with non-smoking controls [4]. ILC subsets have

diverse roles in mucosal inflammation and tissue repair and are activated by cytokines and stress signals [6, 7]. Based on cytokine production and transcription factor expression, ILCs are classified into group 1 ILC (ILC1), ILC2 and ILC3, mirroring the profiles of T-helper (Th) cell subsets [8]. Although ILC precursors (ILCP), ILC1 and ILC2 can be found in human peripheral blood, evidence was provided that under homeostatic conditions ILCs are tissue resident, whereby ILC precursors differentiate locally as a mechanism of maintenance, maturation and tissue adaptation [6, 7, 9–12].

In peripheral blood of COPD patients increased ILC1s and decreased ILC2s were observed, compared with smoking and non-smoking controls [13]. Moreover, ILC1s were positively associated with disease severity as defined by Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification, forced expiratory volume in 1 s (FEV_1) or median number of exacerbations per year. In contrast, the frequency of circulating ILC2s in patients with COPD was negatively associated with disease severity [13]. For asthma there is evidence that ILC2s are increased or more active in peripheral blood and sputum. Because of this association, these cells are thought to contribute to the induction of eosinophilic airway inflammation [14–16].

Recently, subsets of ILC1 and ILC2 were described with specific characteristics regarding inflammatory phenotype or cellular plasticity. Roan *et al*. [17] identified a distinct $CD4^+$ ILC1subset, containing potent producers of tumour necrosis factor-α, granulocyte–macrophage colony-stimulating factor and interleukin (IL)-2, which was increased in patients with the autoimmune disease systemic sclerosis. We identified steroid-resistant $CD45RO^+$ inflammatory ILC2s that were elevated in blood from patients with severe asthma [18]. In addition, c-kit (CD117) expression on a subpopulation of ILC2s was associated with functional plasticity [19, 20].

Given the evidence that ILCs are involved in the pathophysiology of both COPD and asthma, in this report we aimed to compare the inflammatory phenotype of the ILC1 and ILC2 subsets in patients with COPD and asthma, particularly because the distinction between the two conditions may be difficult in clinical practice and because of the coexistence of asthma and COPD in some patients. In our experiments, we used smoking and non-smoking healthy individuals as controls. We investigated peripheral blood ILCs for the expression of markers associated with plasticity or inflammatory phenotypes, including CD4 and CD45RO on ILC1s and c-kit (CD117) and CD45RO on ILC2s, with the primary objective of identifying disease-specific inflammatory ILC profiles in individuals with COPD compared to those with asthma.

Materials and methods

Study design

In this prospective cross-sectional study design, we investigated ILC populations in peripheral blood of patients with COPD and patients with asthma. We included two controls groups: smoking controls and non-smoking controls (figure 1).

Participants

Peripheral blood was obtained from patients and controls included in two clinical studies conducted in the Franciscus Gasthuis and Vlietland Hospital Rotterdam, the Netherlands (NCT03278561 and NL8286, executed between 2017 and 2021). The Medical Ethics Committees United (MEC-U) approved the studies, and all participants provided a written informed consent. The Grandpa study had the following inclusion criteria: patient with COPD or (ex)-smoking individual with age >40 years and >10 pack-years (20 daily cigarettes for 1 year). COPD patients were diagnosed by a pulmonologist based on the presence of airway obstruction, measured by FEV_1 divided by the forced vital capacity (FVC) <0.7; FEV_1 and FVC measurements were performed according to the American Thoracic Society (ATS)/European Respiratory Society (ERS) taskforce “standardisation of spirometry”, with the Vmax SensorMedics Viasys, type 6200 Encore (SensorMedics) [21]. All COPD patients were clinically stable for >6 weeks and did not use a high dose of oral corticosteroids 6 weeks before participation. Severe COPD and asthma patients who used a daily maintenance dose of 5 mg prednisone were eligible for inclusion in the study. Patients with features of both asthma and COPD were excluded. A second cohort, the Grandma study, served as a disease group for comparison. The study also included never- or ex-smoking individuals with <10 pack-years. The (ex)-smoking control individuals included had no (reversible) airway obstruction. Detailed inclusion and exclusion criteria of this study have been described previously [18, 22–24].

Clinical data collection

Clinical data were collected specifically for this prospective cross-sectional study design. All patients underwent spirometry and venous blood sampling and completed questionnaires about quality of life and symptoms. Collected patient characteristics included age, sex, body mass index (BMI), smoking status, number of pack-years, medication use, medical history, family history and start of symptoms.

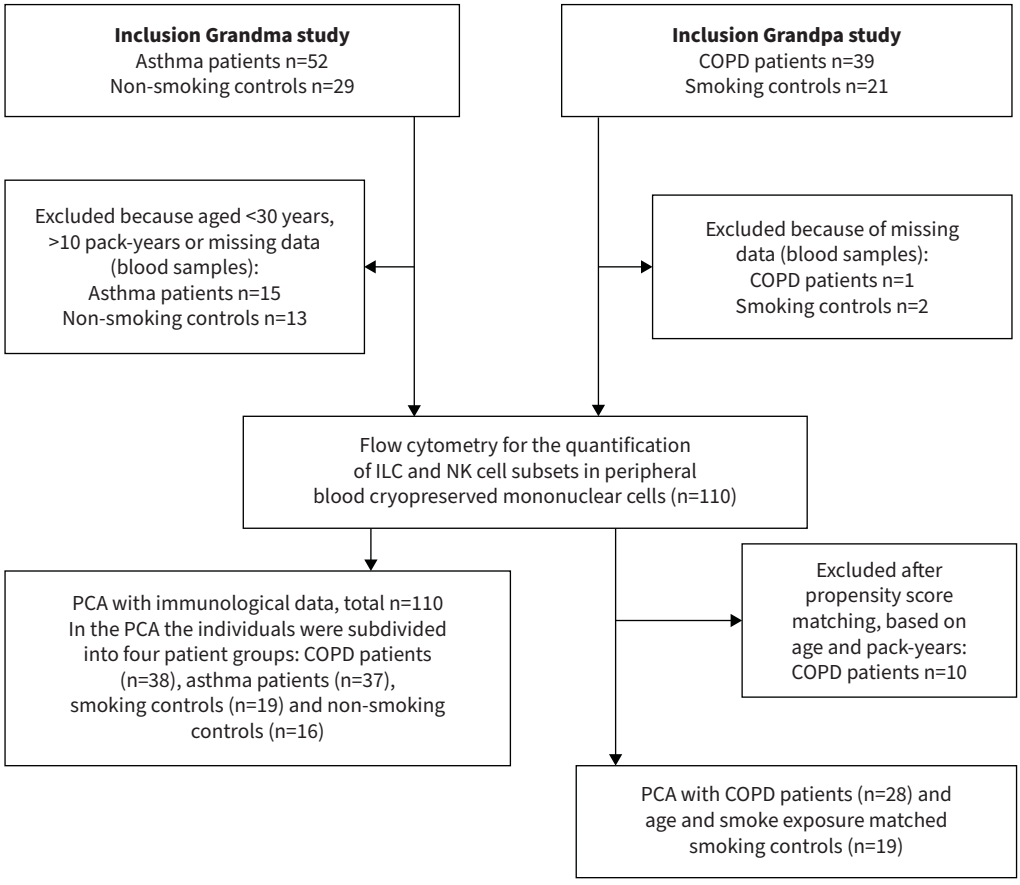

FIGURE 1 Patient enrolment. Details of the Grandma and Grandpa studies are given in the Materials and methods section. ILC: innate lymphoid cells; NK: natural killer; PCA: principal component analysis.

Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from patient or control blood draws, using standard procedures [22], and ILC subsets were characterised by flow cytometry, as previously described [18]. Briefly, cells were stained with fluorochrome-labelled antibodies (supplementary table S1) for 30 min at 4°C in PBS (Gibco) containing 0.5% BSA (Sigma Aldrich) and 2 mM EDTA (Sigma Aldrich). After washing with PBS, a live/dead marker was stained for 15 min at 4°C in PBS. Data were acquired with a Symphony A5 flow cytometer (BD Biosciences, Erembodegem, Belgium) and analysed using FlowJo 10.8.1 software (Tree Star Inc, Ashland, Ore).

Statistical analyses

Principal component analysis (PCA) was performed using R and R-Studio (R-Studio Server Version 1.4.1717) and the packages FactoMineR and Factorextra, as described previously [25]. The number of dimensions to be interpreted were determined by the R package FactoInvestigate. In comparisons of four groups, the Kruskal–Wallis test with Bonferroni correction to account for multiple testing was employed. In cases involving two groups, we utilised the Mann–Whitney U test. To provide the best representation of the data variability, dimensions with an inertia higher than the inertia obtained by a random distribution were considered. After the formation of dimensions by PCA, the diagnoses (COPD, asthma, smoking control or non-smoking control) were labelled, and statistical significance was calculated per dimensions by Kruskall–Wallis test with Bonferroni correction for multiple testing in IBM SPSS Statistics (Version 28.0.0.0 (190)). p-values <0.05 were considered statistically significant. In a sub-analysis of smoking individuals with COPD and without COPD (dependent), a propensity score matching by logistic regression was used to reduce the bias of pack-years and age (predictors). Probabilities were estimated, ranging from 0 to 1, for each patient in the study population. After propensity score matching, a second PCA was performed.

Results

Patient characteristics

From a total of 141 individuals of the COPD and asthma cohorts described in the Materials and methods, 110 patients met the inclusion criteria for our study (for enrolment and selection of patients see figure 1). Characteristics of the COPD patients (n=38), asthma patients (n=37), and smoking (n=19) and non-smoking (n=16) controls are shown in table 1. Quantification of blood leukocyte differentiation showed significantly increased proportions of neutrophils, lymphocytes and monocytes in COPD patients in comparison with asthma patients (supplementary table S2). Neutrophils were also significantly increased in smoking controls in comparison with asthma patients. Eosinophils did not differ between the groups.

COPD patients show increased CD4^{+} and CD4^{-} ILC1s and reduced CD117^{-} ILC2s in peripheral blood compared to asthma patients

To characterise the populations of NK cells and ILCs in peripheral blood of the four groups of patients and controls, we analysed PBMC fractions by flow cytometry. We stained PBMCs with a rich cocktail of antibodies against lineage markers to exclude lineage-positive cells, and quantified NK cells using CD56 and ILC subsets using CD127, CD117 and CRTH2 (see supplementary figure S1 for gating strategy).

NK cells are generally divided into a mainly cytokine-producing CD56^{bright} and a mainly cytolytic CD56^{dim} subset [26]. There were no significant differences between COPD and asthma patients in the proportions of CD56^{bright} NK cells within the lymphocyte gate. However, COPD patients showed significantly reduced proportions of CD56^{bright} NK cells in comparison with non-smoking controls (figure 2a). CD56^{dim} NK cells were not different across the four groups.

TABLE 1 Characteristics of COPD and asthma patients and controls

	COPD	Asthma	Smoking controls	Non-smoking controls	p-value
Particiapants, n	38	37	19	16	
Female	23 (60.5)	21 (56.8)	9 (47.4)	8 (50)	0.729
Age years	60 (56–68)$^{\#}$	58 (44–64)	60 (45–67)	48.5 (40–64.5)$^{\#}$	0.042
BMI kg·m^{-2}	25.1 (22.2–30.0)	27.6 (24.7–31.9)	26.3 (24.6–29.5)	27.3 (23.9–30.5)	0.216
Smoking status					0.020
Never-smoker	0	28 (75.7)	0	13 (81.3)	
Ex-smoker	17 (44.7)	9 (24.3)	12 (63.2)	3 (18.8)	
Current smoker	21 (55.3)¶	0	7 (36.8)¶	0	
Pack-years	33 (27–49)	0 (0–5)	19 (13–30)	0 (0–0)	<0.001^{+}
FEV$_1$ post % pred	48 (31–63)	95 (80.5–106)	100 (86–105)	103.5 (94.3–109.3)	<0.001
FEV$_1$/FVC post % pred	46.0 (35.7–56.7)	77 (70–85.5)	73.9 (70.2–81.1)	84 (77.3–103.5)	<0.001
Exacerbations last year					0.087
0	19 (50)	21 (56.8)	100 (19)	100 (16)	
1	10 (26)	7 (18.9)	0	0	
>1	9 (24)	9 (24.3)	0	0	
Medication					<0.001
SABA	13 (33.3)	7 (18.9)	0	0	
SAMA	5 (12.8)	3 (8.1)	0	0	
LABA	32 (82.1)	38 (97.4)	0	0	
LAMA	34 (87.2)	6 (16.2)	0	0	
ICS	26 (66.6)	39 (100)	0	0	

Data are presented as n (%) or median (interquartile range), unless otherwise stated. Statistically significant differences between COPD patients, asthma patients and any of the control groups, adjusted by the Bonferroni correction for multiple testing. BMI: body mass index; FEV$_1$: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; SABA: short-acting β-agonist; SAMA: short-acting muscarinic antagonist; LABA: long-acting β-agonist; LAMA: long-acting muscarinic antagonist; ICS: inhaled corticosteroid. $^{\#}$: for age, there was a significant difference between COPD and the non-smoking control groups; ¶: for current smoking, there was a significant difference between COPD and smoking controls; $^{+}$: although there were significant differences in pack-years across the four groups, the difference between COPD and smoking control group was not significant (p=0.674).

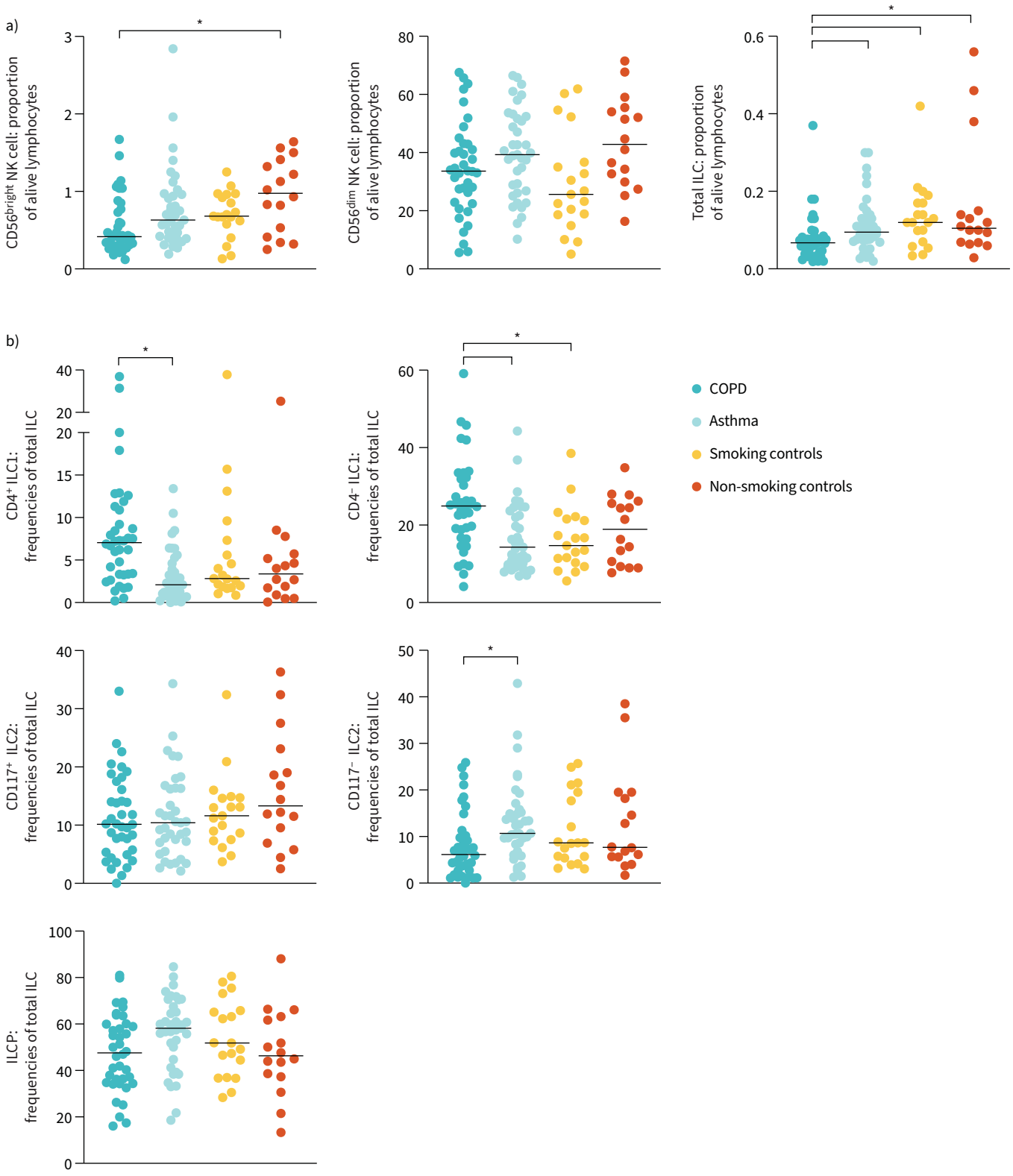

FIGURE 2 Aberrant frequencies of innate lymphoid cells (ILC) in peripheral blood of COPD patients. Subsets of peripheral blood ILCs of COPD patients, asthma patients, and smoking and non-smoking controls, as determined by flow cytometry. a) Proportions of natural killer (NK) cells and total ILCs within the alive lymphocyte population. b) Frequencies of the indicated ILC subsets within the total ILC population. Dots represent an individual patient or control, as indicated. Horizontal bars represent median values. Statistical analyses were performed using Kruskal–Wallis test combined with a Bonferroni correction for multiple testing. *p<0.05.

The proportions of total ILCs were significantly lower in COPD patients in comparison with asthma and the two control groups (figure 2a). In COPD patients the frequency of $CD4^+$ ILC1s within the total ILC population appeared to be higher compared with all three other groups, although significance was only reached for the comparison with asthma patients (figure 2b). The frequency of $CD4^-$ ILC1s was also higher in COPD patients than in smoking controls and asthma patients (figure 2b).

The proportions of $CD117^+$ ILC2s, which display a high level of plasticity towards ILC1 and ILC3 [19, 20], were similar across the four groups of patients and controls (figure 2b). In contrast, the subset of $CD117^-$ ILC2s was significantly reduced in COPD patients, compared with asthma patients, but not when compared with controls (figure 2b). Finally, the proportions of ILC precursors ($CD117^+CRTH2^-$ ILCs [9]) did not differ between the four groups.

Taken together, these analyses revealed various significant differences in the frequencies of NK cells and ILCs in peripheral blood between COPD patients, asthma patients and controls. As summarised in supplementary figure S2, within the total population of circulating ILCs of COPD patients, both $CD4^+$ and $CD4^-$ ILC1 were elevated and $CD117^-$ ILC2s were decreased, when compared to asthma patients. Proportions of $CD4^-$ ILC1s in COPD patients were also significantly higher than in smoking controls.

COPD patients show a shift from $CD45RA^+$ to $CD45RO^+$ cells in $CD4^-$ ILC1s and $CD117^-$ ILC2s

Since we recently showed that $CD45RO^+$ ILC2s have an inflammatory phenotype and are increased in the circulation of patients with severe, uncontrolled asthma [18], we decided to analyse surface expression of the CD45RA and CD45RO isoforms, both on ILC1s and ILC2s.

The frequencies of $CD45RA^+$ and $CD45RO^+$ cells within the $CD4^+$ ILC1 population were variable and did not differ significantly across the four groups (figure 3a). In contrast, a significant decrease in the fraction of $CD45RA^+$ cells was seen within the $CD4^-$ ILC1 population of COPD patients, when compared with asthma patients. Conversely, we observed a parallel increase in the proportion of $CD45RO^+$ $CD4^-$ ILC1, although this did not reach statistical significance (figure 3a).

COPD patients displayed decreased proportions of $CD45RA^+$ cells and increased proportions of $CD45RO^+$ cells in the $CD117^-$ ILC2 population, which was significant when compared to patients with asthma (figure 3b). Next to our aim to compare the inflammatory phenotypes of asthma and COPD, we conducted additional analyses to compare COPD patients directly with the smoking controls that did not develop COPD. Using a Mann–Whitney U test to compare the two groups, we observed that COPD patients had significantly lower proportions of $CD45RA+CD117^+$ ILC2s ($p=0.005$) and $CD45RA+CD117^-$ ILC2s ($p=0.006$) than smoking controls (figure 3b). Furthermore, a trend was observed that inflammatory $CD45RO^+$ ILC2s were more prevalent in patients with COPD, but this did not reach significance.

Correlation analysis showed that COPD patients with elevated proportions of $CD45RO^+$ cells within the population of $CD117^-$ ILC2s also had increased proportions of $CD45RO^+$ within $CD117^+$ ILC2s or $CD4^-$ ILC1s, but these correlations were weak (supplementary figure S3). We did not find correlations between proportions, subsets or phenotypes of ILC1s or ILC2s and clinical parameters such as FEV_1, GOLD stage or the number of exacerbations in the previous year.

In summary, particularly when compared with patients with asthma, both $CD4^-$ ILC1 and $CD117^-$ ILC2 from COPD patients manifested a shift from surface $CD45RA^+$ to $CD45RO^+$ expression, which for ILC2s was shown to be associated with an inflammatory phenotype [18].

PCA separates COPD patients from asthma patients on the basis of ILC1 and ILC2 characteristics

To obtain a more comprehensive overview of the differences between ILC subsets across the four patient and control groups, we performed a dimensionality reduction by PCA (figure 4a). Hereby, we included frequencies and phenotypes of ILC subsets, as well as peripheral blood leukocyte differentiation parameters. The distribution over dimension 1 (Dim.1) and Dim.2 was non-random, which was not due to age, sex and smoking status (supplementary figure S4). COPD patients were significantly separated from asthma patients, based on both Dim.1 and Dim.2 (with 18.7% and 11.7% of variance, respectively) (figure 4b). Dim.1 showed a significant difference between COPD patients and all the other groups. COPD patients and smoking controls could not be separated based on Dim.2, implying that Dim 2 was more related to smoking than to the disease. Likewise, Dim.2 did not separate asthma patients from non-smoking controls. Whereas the subsets of $CD117^-$ ILC2s and $CD4^-$ ILC1s contributed most to Dim.1, Dim.2 was dominated by the absolute numbers of leukocytes, neutrophils and the subsets of $CD4^+$ ILC1s (figure 4c).

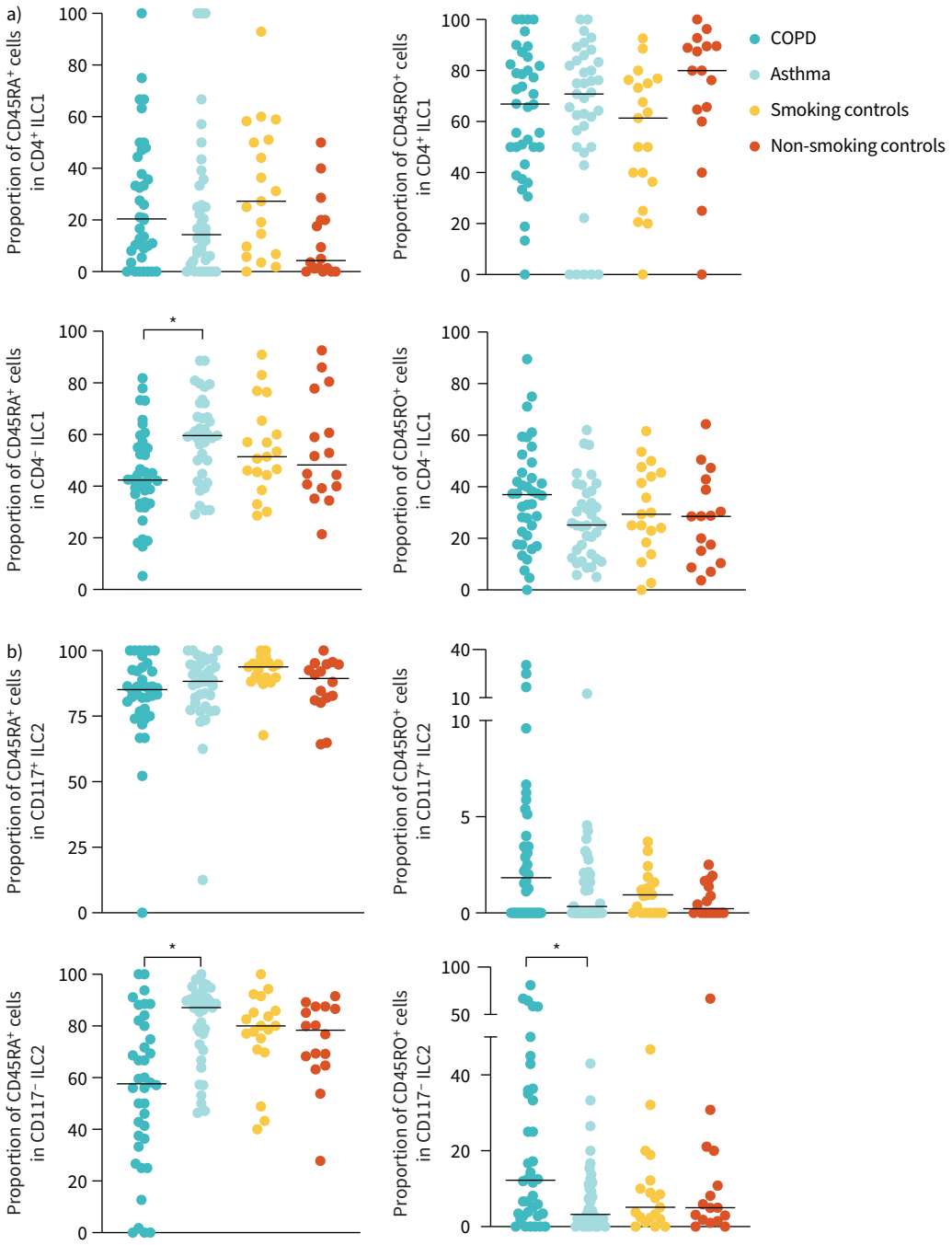

FIGURE 3 Aberrant phenotype of innate lymphoid cells (ILC) in peripheral blood of COPD patients. Distribution of CD45RA$^+$ and CD45RO$^+$ subsets of ILCs in peripheral blood of COPD patients, asthma patients, smoking and non-smoking controls, as determined by flow cytometry within a) CD4$^+$ ILC1 and CD4$^-$ ILC1 subsets, and b) CD117$^+$ ILC2 and CD117$^-$ ILC2 subsets. Dots represent an individual patient or control, as indicated. Horizontal bars represent median values. Statistical analyses were performed using Kruskal–Wallis test combined with a Bonferroni correction for multiple testing. *: p<0.05.

In summary, this PCA revealed that COPD patients were significantly separated from asthma patients, smoking controls and non-smoking controls (independent of age or sex) on the basis of the phenotype of CD117$^-$ ILC2s and CD4$^-$ and CD4$^+$ ILC1s. The contribution of NK cells, CD117$^+$ ILC2s and ILCP to the separation of COPD patients was limited.

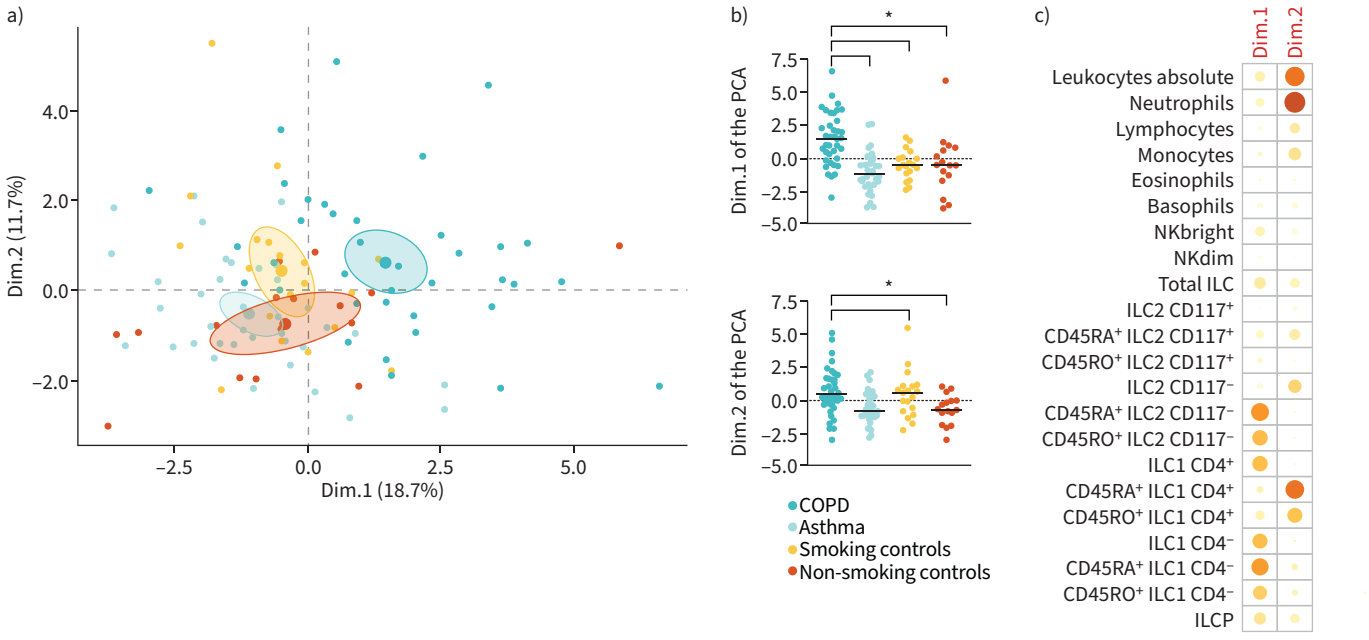

FIGURE 4 Principal component analysis (PCA) distinguishes COPD patients from control groups. PCA of frequencies and phenotype of peripheral blood innate lymphoid cells (ILC) subpopulations and leukocyte differentiation in peripheral blood of COPD patients, asthma patients, and smoking and non-smoking controls. a) PCA in which each symbol point represents an individual patient or control. b) Dimension 1 (Dim.1) and 2 (Dim.2) of PCA from the indicated patients and control groups. c) Relative contribution (as shown by size and orange colour range of dots) of the indicated ILCs and leukocyte subgroups in Dim.1 and Dim.2 of the PCA. Statistical analyses were performed using Kruskal–Wallis test combined with a Bonferroni correction for multiple testing. *: p<0.05. ILCP: ILC precursors; NK: natural killer.

ILC subsets or phenotype characteristics do not separate ex-smokers from current smokers

To allow for a comparison of COPD patients and smoking controls with similar smoke exposure and age, a propensity score matching was performed. Upon exclusion of 10 COPD patients (figure 1), there were no significant differences between the COPD patients (n=28) and smoking controls (n=19) in sex, age, pack-years and current smoking status (supplementary table S3).

When we performed a PCA using these individuals, we observed that the distribution over Dim.1 and Dim.2 was independent of sex and age (supplementary figure S5). Interestingly, our immune cell parameters separated ex-smokers from current smokers in Dim.2 (supplementary figure S6A,B). In Dim.1 the frequency values of various ILC1subsets were dominant; Dim.2 was dominated by absolute leukocyte numbers, and frequencies of neutrophils as well as frequencies of total ILCs (supplementary figure S6C). Although values for COPD patients and controls tended to be different in Dim.1, significance was not reached, likely due to small groups sizes (supplementary figure S6).

In summary, our analysis revealed that frequencies of neutrophils and total ILCs, as well as absolute leukocyte numbers separated ex-smokers and current smokers, but parameters of ILC subset phenotype or subsets did not.

Discussion

In this translational study we investigated whether the frequency or inflammatory phenotype of peripheral blood ILC subsets differ between COPD patients and asthma, smoking controls and non-smoking controls, when adjusted for age, sex, smoking status and pack-years. We observed that COPD patients had higher frequencies of both $CD4^+$ and $CD4^-$ ILC1s within the total ILC population when compared with controls, which reached significance for the comparison between COPD and asthma patients. In addition, proportions of $CD117^-$ ILC2s, representing the most mature population of ILC2s [19, 20], were significantly reduced. Both $CD4^-$ ILC1s and $CD117^-$ ILC2s manifested a shift from CD45RA to CD45RO surface expression, which for ILC2s was shown to be associated with an inflammatory phenotype in severe asthma [18]. Finally, PCA separated COPD patients from smoking and non-smoking controls and asthma patients. Hereby, mainly the $CD4^-$ and $CD4^+$ ILC subsets as well as $CD117^-$ ILC2s contributed to the variance in the first two dimensions, and not so much the ILC precursors or the more plastic $CD117^+$ subpopulation of ILC2s.

Our results are in line with Silver *et al*. [27], who also found elevated proportions of ILC1 and reduced proportions of ILC2 in COPD patients, which were associated with disease severity. However, we did not observe correlations between proportions, subsets or phenotype of ILCs in COPD patients and key clinical parameters. The relative frequencies of ILC1 and ILC2 subsets in the circulation of COPD patients seem to reflect local changes in the lung. It was reported that ILC1 frequencies are increased in the lungs of COPD patients and correlate with smoking and severity of respiratory symptoms [28, 29]. Also, proportions of pulmonary ILC2s were lower in patients with severe, GOLD stage IV, COPD than in GOLD stage I or II patients or healthy controls [28], and the frequency of IL-13$^+$ ILC was positively correlated with FEV_1 [29]. Accordingly, it was shown in mice that whereas proportions of ILC1s and ILC3s in the lung increased following smoke exposure, ILC2s showed a decrease [30]. Nevertheless, Blomme *et al*. [29] reported an increase in both ILC1s and ILC2s in bronchoalveolar lavage in mice exposed to cigarette smoke. Although evidence was provided that ILC3 levels are elevated in donor COPD lungs and smoker lungs in comparison with controls [31–33], this cannot be correlated with peripheral blood since circulating ILC3 are essentially absent [9].

We found that the frequency of CD117$^-$ ILC2s in COPD patients was lower than in asthma patients, but that CD117$^+$ ILC2s were comparable. This implies that particularly the more committed CD117$^-$ ILC2s with a greater potential to produce type 2 cytokines are maintained, rather than the more immature and plastic CD117$^+$ ILC2 [19, 20]. Our finding of elevated frequencies within the ILC2 population of CD45RO$^+$ inflammatory cells, which were associated with corticosteroid resistance in asthma patients [18], may suggest that these cells are also involved in steroid resistance in COPD patients.

Although CD4$^+$ ILC1s were recognised as a distinct ILC1subset increased in systemic sclerosis, it is of note that there are concerns about the identity of ILC1s, which may reflect technical issues related to contaminating T-cells [17, 19, 34]. To minimise contamination of ILC1, we included antibodies to CD3, TCRαβ and TCRγδ in our lineage marker cocktail. In line with Roan *et al*. [17] we found that ∼70% of the CD4$^+$ ILC1s were CD45RO$^+$ and ∼30% of CD4$^-$ ILC1 were CD45RO$^+$. Literature on the inflammatory characteristics of CD45RO$^+$ ILC1 is currently lacking. Nevertheless, the finding of increased CD4$^+$ ILC1s in systemic sclerosis suggests that CD4$^+$ ILC1s, of which a majority expresses CD45RO, are pro-inflammatory in nature. It is attractive to speculate that the relative shift from CD45RA$^+$ to CD45RO$^-$ CD4$^-$ ILC1s in COPD patients, compared to asthma patients, may point to a pro-inflammatory role of these cells in COPD. Future experiments should show the functional implications of surface CD4 and CD45RO expression on ILC1s.

Our study has some limitations. Firstly, we quantified the proportions of ILCs and not their functional properties, such as cytokine production. This is also relevant in the light of conflicting data regarding the identity of ILC1s (as described above; see Simoni and Newell [34]) and the recent identification of unconventional CRTH2$^-$ ILC2s [35]. Secondly, it remains unknown how the phenotype or activation status of peripheral blood ILCs is linked to pathophysiological effects of these cell populations in the lungs, particularly since ILCs are known to adopt tissue-specific functional phenotypes [36] and ILCs are thought to be largely tissue-resident cells [9, 10]. Thirdly, our study is cross-sectional, and it would be interesting to investigate ILC dynamics over time and during disease exacerbations. Finally, our analyses were explorative with small sample sizes and need to be confirmed in larger, well-defined cohorts of patients.

In conclusion, we provide evidence that COPD patients can be distinguished from asthma patients, as well as smoking and non-smoking controls, by analysing the composition and phenotypic characteristics of the circulating ILC1 and ILC2 compartments. Although the frequencies of mature CD117$^-$ ILC2s were reduced in COPD patients, compared with asthma patients, the fraction of inflammatory CD45RO$^+$ cells within this ILC2 population was significantly increased. These observations suggest that not only ILC1s [13], but also ILC2s may play a role in COPD pathogenesis. Our detailed characterisation of ILC subsets also allowed us to differentiate between COPD inflammation and smoking effects, which may have implications for patient stratification. Further experiments in larger patient cohorts should show whether the shift from CD45RA to CD45RO in ILC1s and ILC2s in COPD is linked to steroid resistance or disease exacerbation, which may prove useful for therapeutic strategies.

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This study is registered at www.clinicaltrials.gov with identifier number NCT03278561 and www.trialregister.nl with identifier number NL8286. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics statement: Peripheral blood was obtained from patients and controls included in two clinical studies conducted in the Franciscus Gasthuis and Vlietland Hospital Rotterdam, the Netherlands (NCT03278561 and NL8286, executed between 2017 and 2021). The Medical Ethics Committees United approved the studies and all participants provided written informed consent.

Author contributions: C.M. van Zelst, J.C.C.M. in 't Veen, L. Krabbendam, R.W. Hendriks and G-J. Braunstahl designed the study; C.M. van Zelst, J.C.C.M. in 't Veen, L. Krabbendam, G-J. Braunstahl, M.J.W. de Bruijn, M. van Nimwegen, D. van Uden, R. Stadhouders, G.A. Tramper-Stranders, R.W. Hendriks and G-J. Braunstahl acquired, analysed or interpreted the of data; C.M. van Zelst, R.W. Hendriks and G-J. Braunstahl wrote the manuscript with input from all authors. All authors concurred with the submission of the manuscript.

Conflict of interest: All authors declare that they have no conflicts of interest related to the topic.

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