Increased cytotoxic T-cells in the airways of adults with former bronchopulmonary dysplasia

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Shareable abstract (@ERSpublications)
Young adults with former BPD display more cytotoxic T-cells in the airways than healthy subjects. These T-cells correlate with FEV_1. Thus, cytotoxic T-cells may contribute to the pathology behind chronic airway obstruction in adults with former BPD. https://bit.ly/3soI4lK

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Abstract

Rationale Bronchopulmonary dysplasia (BPD) in preterm-born infants is a risk factor for chronic airway obstruction in adulthood. Cytotoxic T-cells are implicated in COPD, but their involvement in BPD is not known.

Objectives To characterise the distribution of airway T-cell subsets in adults with a history of BPD.

Methods Young adults with former BPD (n=22; median age 19.6 years), age-matched adults born preterm (n=22), patients with allergic asthma born at term (n=22) and healthy control subjects born at term (n=24) underwent bronchoalveolar lavage (BAL). T-cell subsets in BAL were analysed using flow cytometry.

Results The total number of cells and the differential cell counts in BAL were similar among the study groups. The percentage of $CD3^+CD8^+$ T-cells was higher (p=0.005) and the proportion of $CD3^+CD4^+$ T-cells was reduced (p=0.01) in the BPD group, resulting in a lower CD4/CD8 ratio (p=0.007) compared to the healthy controls (median 2.2 *versus* 5.3). In BPD and preterm-born study subjects, both $CD3^+CD4^+$ T-cells (r_s=0.38, p=0.03) and CD4/CD8 ratio (r_s=0.44, p=0.01) correlated positively with forced expiratory volume in 1 s (FEV_1). Furthermore, $CD3^+CD8^+$ T-cells were negatively correlated with both FEV_1 and FEV_1/forced vital capacity (r_s= −0.44, p=0.09 and r_s= −0.41, p=0.01, respectively).

Conclusions Young adults with former BPD have a T-cell subset pattern in the airways resembling features of COPD. Our findings are compatible with the hypothesis that $CD3^+CD8^+$ T-cells are involved in mechanisms behind chronic airway obstruction in these patients.

Introduction

Bronchopulmonary dysplasia (BPD) is a common cause of respiratory insufficiency in children born very to extremely preterm. Approximately 10–30% of infants born before gestational week 30 with birthweight <1000 g will develop BPD [1, 2]. The pathophysiology of BPD is often linked to underdeveloped lungs, inflammation, barotrauma and volutrauma resulting from mechanical ventilation, and oxidative stress [3, 4]. The precise consequences of BPD in adult age are largely unknown, but there is a risk of developing chronic lung disease, including airway obstruction [5–8]. This patient group is expected to increase, since more survivors of BPD are reaching adult life [9].

COPD is characterised by airway obstruction, presumably caused by inflammation and subsequent remodelling in the airways and lung parenchyma [10–12]. The inflammatory process is known to include

the accumulation of neutrophils, eosinophils, macrophages and/or lymphocytes to a varying degree, depending on subphenotype [10–12]. In COPD, this process is also associated with a pattern of elevated levels of $CD8^+$ T-cells and a lower CD4/CD8 ratio, as indicated in peripheral blood, induced sputum and bronchoalveolar lavage (BAL) [13–16].

It is unknown if there is a persistent inflammation in the airways in adults born prematurely with a history of BPD and, if so, how this is related to lung function. A decrease in the absolute number and proportion of $CD4^+$ T-cells in blood has been shown in preterm-born infants who developed BPD compared to preterm-born infants without BPD [17]. The lower proportion of $CD4^+$ T-cells and the reduced CD4/CD8 ratio seems to persist in the blood into school age in children born preterm, and a weak association to peak expiratory flow has been observed [18]. Studies on BAL fluid from the neonatal period are scarce, but an elevated percentage of neutrophils and an increase in interleukin 8 in infants who developed BPD has been demonstrated [19].

In the present study, we studied pulmonary immune cells and signs of peripheral airway inflammation in adults born prematurely with and without BPD and compared it with patients with asthma and healthy control subjects. Specifically, we investigated major T-cell subsets in BAL to address their potential involvement in airway inflammation and the development of airway obstruction in individuals with a history of BPD.

Materials and methods

More details on the participants and methods are provided in supplementary materials and methods and supplementary tables.

Participants

This case–control study was conducted on subjects from the Lung Obstruction in Adulthood of Prematurely Born study (LUNAPRE; clinicaltrials.gov identifier NCT02923648). Data on lung function and symptoms have been described previously [20]. 22 subjects in the BPD group, 22 in the preterm group, 22 in the asthma group, and 24 healthy controls underwent bronchoscopy and BAL (supplementary table E1; supplementary figure E1). All participants provided written informed consent, and the study was approved by the Swedish Ethical Review Authority (ref: 201211872-31/4) [20]. The diagnosis of BPD was based on the need for supplemental oxygen for ⩾28 days and severity degree determined at 36 weeks' gestational age according to Jobe and Bancalari [21]. All participants were nonsmokers, without ongoing anti-inflammatory treatment (including inhaled or oral corticosteroids, leukotriene receptor antagonists and antihistamines), without respiratory tract infections for ⩾3 months prior to inclusion.

Lung function

Dynamic spirometry with reversibility testing, static lung volumes, diffusing capacity of the lung for carbon monoxide (D_{LCO}), methacholine challenge test and fractional exhaled nitric oxide (F_{eNO}) was performed according to American Thoracic Society/European Respiratory Society guidelines [22–25]. All measures reported for dynamic spirometry are post-bronchodilator.

Peripheral blood analyses

Blood samples were obtained at an outpatient visit before bronchoscopy. Leukocytes with differential counts, markers of systemic inflammation and sensitisation towards airborne allergens were analysed using routine methods at the laboratory of Karolinska University Hospital (Solna, Sweden).

Bronchoscopy and bronchoalveolar lavage

Bronchoscopy and BAL were performed according to a standardised protocol [26–28].

The BAL fluid recovery was quantified, debris and mucus were removed by filtration and the cells were separated by centrifugation. Differential cell counts were determined on cytospin slides prepared with native pellet, stained with May–Grünwald–Giemsa, counting 500 cells per subject.

Immunofluorescence staining and flow cytometry

Lymphocyte subsets in BAL were analysed using flow cytometry (FACS Fortessa; BD Biosciences), processed in FACSDiva 6.1.2 (BD Biosciences) and analysed using FlowJo 10.7.1 (BD Biosciences). Three different panels of monoclonal antibodies were used to describe the major lymphocyte subsets and T-cell differentiation (figure 1a–c, supplementary table E2).

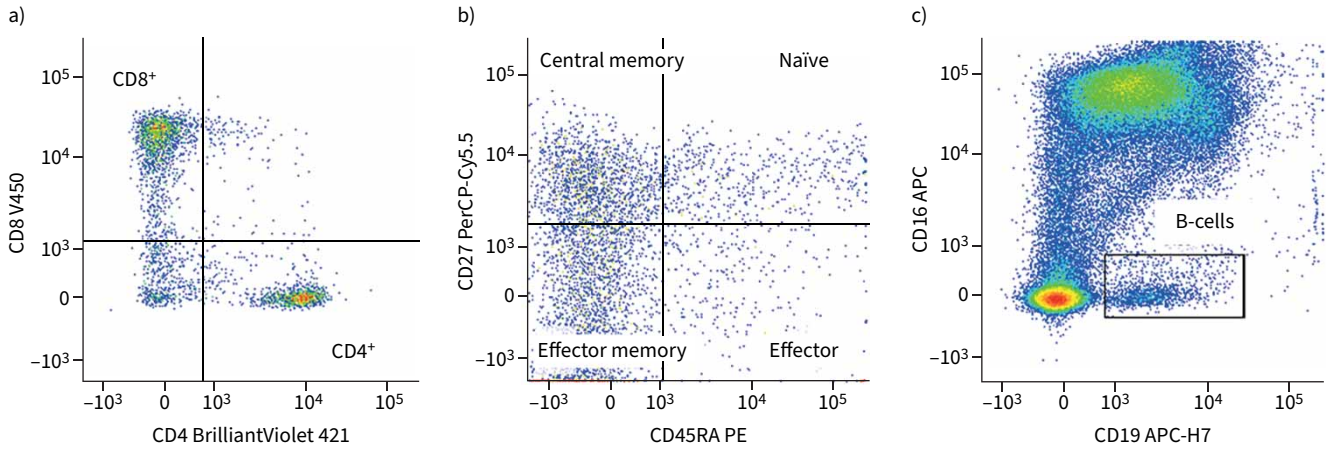

FIGURE 1 Representative flow cytometry dot plots of a) $CD8^+$ (y-axis) and $CD4^+$ (x-axis) among $CD3^+$ T-cells from bronchoalveolar lavage (BAL); b) T-cell differentiation among $CD3^+$ T-cells from BAL; and c) B-cells among live cells from BAL. Samples were taken from three different study subjects. PE: phycoerythrin; PerCP: peridin chlorophyll protein; Cy: cyanine dye; APC: allophycocyanine.

ELISA

Protein concentrations of Granzyme B and perforin in BAL fluid were quantified using commercial ELISA kits, including Human Granzyme B DuoSet (R&D Systems) and Human Perforin ELISA Set (Diaclone), respectively, following the manufacturer's protocol.

Statistical methods

Demographic data are presented as median (range) for continuous variables, or n (%) for categorical variables. Comparisons between the groups were performed using the Kruskal–Wallis test with *post hoc* analysis using the Mann–Whitney U-test. Pearson's Chi-squared and Fisher's exact tests were used when appropriate, with correction for multiple hypothesis testing using false discovery rate (FDR) according to Benjamini–Hochberg. Statistical significance was determined as $p<0.05$ and FDR<0.30 [29, 30]. Correlations were assessed with Spearman's test. Analyses were performed with Stata 13.1 (StataCorp), or in R 4.0.3.

Results

Participants

Perinatal characteristics and demographic characteristics of the adult study population are presented in supplementary table E1 and table 1. Both gestational age and birthweight were lower in the BPD group compared to the preterm group; median 26 (24–31) weeks *versus* 29.5 (27–32) weeks and median 960 (583–2136) g *versus* 1480 (710–2200) g. The two preterm-born groups were slightly younger at the time of inclusion; median 19.6 years in the BPD group and 19.1 years in the preterm group compared to 20.3 years and 20.5 years in the asthma and healthy control groups, respectively (table 1). Only four in the preterm group and 18 in the BPD group were <28 weeks and/or <1000 g. A subgroup analysis was performed on these individuals (supplementary table E3) and we made correlation analyses of both gestational age and birthweight to lung function and T-cell subsets without any statistically significant results (data not shown).

Lung function

The BPD group had lower FEV_1 and FEV_1/FVC compared to the other three study groups (table 1), as reported previously for the full cohort [20]. 68% of the BPD group and 59% of the preterm group were positive in the methacholine challenge test. According to the study inclusion and exclusion criteria, all patients with asthma, but none of the healthy control subjects had a positive methacholine challenge test. D_{LCO} was decreased in both preterm groups compared to the healthy control group, and was lower in the BPD group compared to the asthma group. Patients with asthma had increased F_{eNO} compared to healthy control subjects and the BPD group, but not compared to the preterm group (table 1).

Peripheral blood cells, markers of systemic inflammation and sensitisation towards airborne allergens

The asthma group had elevated levels of blood eosinophils compared to the healthy control ($p=0.002$), preterm ($p=0.0001$) and BPD groups ($p=0.02$), respectively (supplementary table E4). No differences were

TABLE 1 Characteristics of study participants

	Healthy	Asthma	Preterm	BPD
Participants	24	22	22	22
Male/female	12/12	9/13	10/12	11/11
Age (years), median (range)	20.5 (18.3–23.8)	20.3 (18.6–23.3)	19.2* (18.7–22.4)	19.6* (18.2–21.2)
BMI (kg·m^{-2}), median (range)				
Male	21.6 (19.7–32.5)	23.9 (20.6–29.4)	20.3 (16.8–26.7)	20.4 (17.9–29.8)
Female	21.8 (18.1–37.9)	21.2 (18.2–34.2)	22.1 (17.0–27.7)	22.1 (17.9–32.6)
Post-bronchodilator FEV_1 (% pred)	108.9 (99.7–114.1)	101.8 (94.1–109.0)	102.8 (96.9–113.3)	85.0*** (76.2–99.1)
Post-bronchodilator FEV_1 (z-scores)	0.78 (−0.03–1.26)	0.15 (−0.52–0.77)	0.24 (−0.27–1.18)	−1.31*** (−2.00–−0.08)
Post-bronchodilator FEV_1/FVC	0.90 (0.86–0.93)	0.86 (0.83–0.89)	0.93 (0.89–0.95)	0.80*** (0.74–0.88)
Post-bronchodilator FEV_1/FVC (z-scores)	0.42 (−0.26–0.89)	−0.19* (−0.43–0.32)	0.87 (0.14–1.33)	−1.04*** (−1.86–0.18)
Post-bronchodilator reversibility (%)	4.9 (2.0–6.3)	5.9* (3.6–8.3)	3.0 (1.8–7.6)	6.9* (4.3–11.9)
Post-bronchodilator reversibility (positive)[#]	0	2 (9)	2 (9)	5* (23)
D_{LCOadj} (% pred)	86.5 (80–96)	81.5 (75–91)	73*** (65–83)	70*** (64–73)
RV (% pred)	84.5 (68.5–105.5)	72.5 (52–96)	82 (65–132)	87 (60–98)
RV/TLC	19 (17–22.5)	18.5 (14–23)	21 (18–23)	20 (17–24)
F_{eNO} (ppb)	10.9 (9.7–17.1)	31.4** (11.6–49.2)	13.3 (11.0–19.8)	13.4 (9.2–21.8)

Data are presented as n, median (interquartile range) or n (%), unless otherwise stated. BPD: bronchopulmonary dysplasia; BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; D_{LCOadj}: diffusing capacity of the lung for carbon monoxide adjusted for blood haemoglobin; RV: residual volume; TLC: total lung capacity; F_{eNO}: fractional exhaled nitric oxide. #: defined as increase in FEV_1 of 12% and 200 mL. *: p<0.05, **: p<0.01, ***: p<0.001, comparing BPD, preterm and asthma groups to healthy control group.

observed between the four study groups regarding the acute-phase proteins. Eight out of 22 in the preterm group, but only two out of 22 in the BPD group (p=0.03) were sensitised to common airborne allergens as measured by Phadiatop. All patients in the asthma group, but none of the healthy control subjects were positive, according to the inclusion criteria.

BAL cell counts

The median (interquartile range) percentage of recovery from BAL fluid was 74.0% (68.0–78.0%); it was significantly lower in the asthma group compared to the preterm and BPD groups (table 2). Total cell yield and cell concentration were 17.2 (14.6–22.1) million cells and 101.1 (78.7–121.3) million cells·L^{-1}, respectively, and did not differ between the groups. The predominant cell populations in BAL were macrophages (90.8%, 84.4–94.2%) and lymphocytes (8.0%, 5.0–14.6%). We observed no statistically significant differences in BAL leukocyte differential counts between the four study groups, except for the asthma group, which had a higher percentage of eosinophils compared to the BPD group.

Flow cytometry of BAL lymphocytes

Univariate statistics for all comparisons, along with correction for multiple testing using FDR, and effect sizes are presented in supplementary table E5.

TABLE 2 Bronchoalveolar lavage (BAL) characteristics of study participants

	Healthy	Asthma	Preterm	BPD
Performed BAL	24	22	22	22
Recovery (%)	75.5 (70.0–80.0)	70.0 (62.0–78.0)	76.5** (71.0–81.0)	75.0* (66.5–79.5)
Total cell yield (×10^6)	19.6 (16.3–25.5)	15.6 (14.9–18.7)	19.6 (15.0–26.2)	17.0 (11.8–19.2)
Cell concentration (×10^6 cells·L^{-1})	110.3 (86.5–130.6)	92.3 (82.3–115.5)	102.3 (78.0–182.5)	94.5 (70.3–113.9)
Differential count for leukocytes				
Macrophages (%)	89.6 (84.8–94.2)	92.0 (85.4–93.6)	90.7 (79.1–94.7)	91.0 (82.6–95.2)
Lymphocytes (%)	8.5 (4.8–15.2)	6.6 (5.4–11.8)	7.9 (5.1–19.6)	9.0 4.5–16.6)
Neutrophils (%)	0.4 (0.0–1.0)	0.8 (0.0–1.0)	0.8 (0.0–1.0)	0.4 (0–0.7)
Eosinophils (%)	0.0 (0.0–0.2)	0.4 (0.0–1.0)	0.0 (0.0–0.4)	0 (0.0–0.0)**
Basophils (%)	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.0 (0.0–0.4)	0.0 (0.0–0.0)
Mast cells per 10 fields of vision	0.0 (0.0–1.0)	0.0 (0.0–2.0)	0.0 (0.0–0.0)	0.0 (0.0–1.0)

Data are presented as n or median (interquartile range). BPD: bronchopulmonary dysplasia. *: p<0.05, **: p<0.01, comparing BPD and preterm group to asthma group.

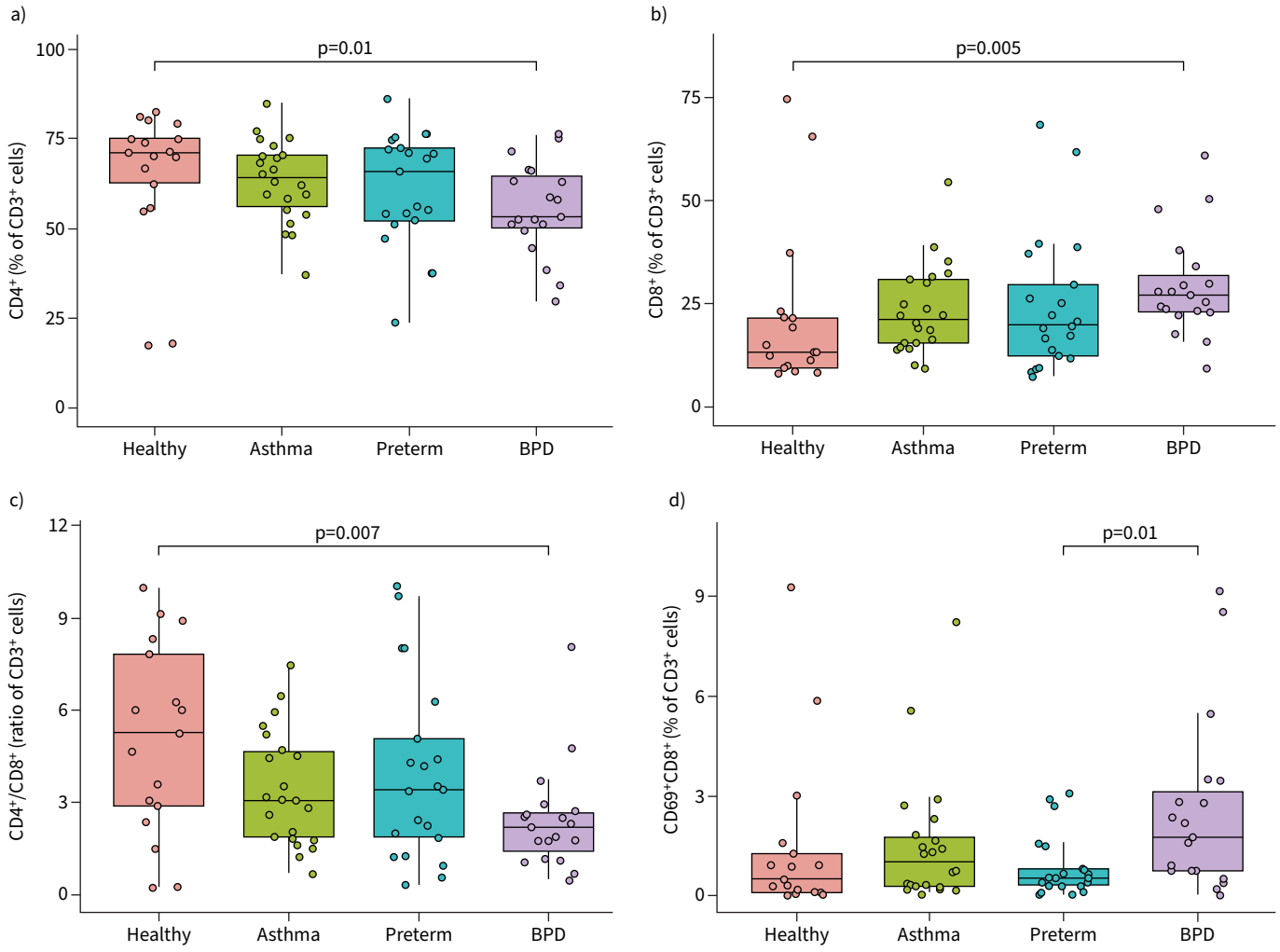

FIGURE 2 Dot plots show the percentage in bronchoalveolar lavage $CD3^+$ cells, with boxes demonstrating interquartile range, horizontal line indicating the median, and whiskers showing the 95% confidence interval. a) $CD3^+CD4^+$ T-cells (T-helper cells) (%); b) $CD3^+CD8^+$ T-cells (cytotoxic T-cells) (%); c) CD4/CD8 ratio of $CD3^+$ cells; d) $CD3^+CD8^+CD69^+$ (activated cytotoxic T-cells) (%). All cells were gated for live cells. Analysis with Mann–Whitney U-test. BPD: bronchopulmonary dysplasia.

$CD4^+$ T-cells

The percentage of $CD4^+$ T-cells was reduced in BAL from the BPD group compared to the healthy control group (p=0.01; figure 2a). The result persisted when the pooled preterm (BPD and preterm) and the healthy control group were compared (p=0.04).

$CD8^+$ T-cells

The percentage of $CD8^+$ T-cells was higher in BAL from the BPD as well as in the pooled preterm group compared to the healthy control group (p=0.005, p=0.03, respectively; figure 2b).

CD4/CD8 ratio

The CD4/CD8 ratio in BAL from the BPD group was lower compared to the healthy control group (p=0.007; figure 2c).

Activated T-cells

We targeted CD69 as a marker for $CD4^+$ and $CD8^+$ T-cell activation. The preterm group had a lower proportion of $CD4^+CD69^+$ T-cells compared to the healthy control group (p=0.01; supplementary figure E2a). The BPD group had an increased proportion of $CD8^+CD69^+$ T-cells when compared to the preterm group (p=0.01; figure 2d).

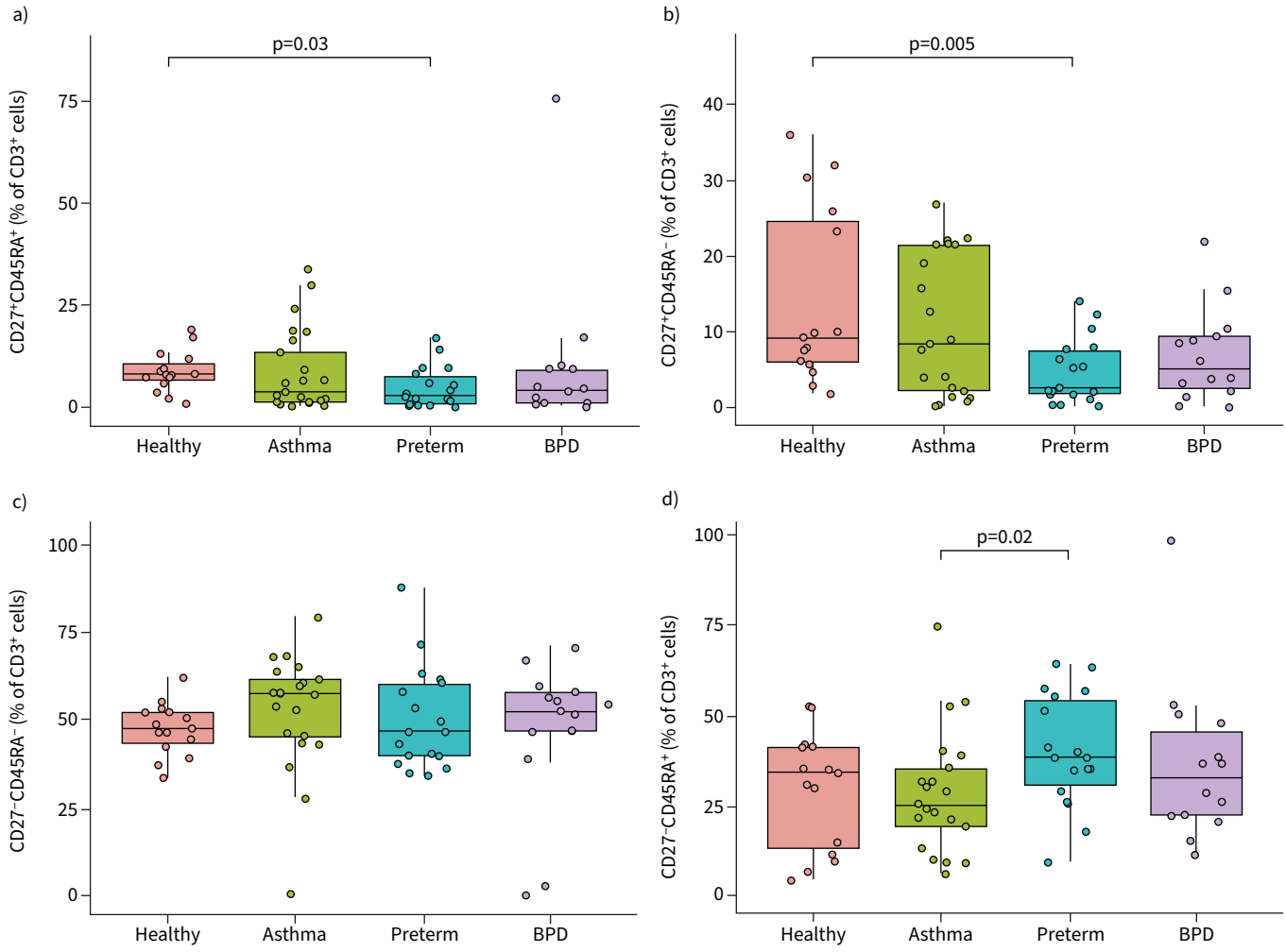

FIGURE 3 Characterisation of T-cell differentiation in bronchoalveolar lavage $CD3^+$ cells. The dot plots show the percentage, and the boxes demonstrate interquartile range with the horizontal line for median for a) $CD27^+CD45RA^+$ (naïve cells) (%); b) $CD27^+CD45RA^-$ (central memory cells) (%); c) $CD27^-CD45RA^-$ (effector memory cells) (%); and d) $CD27^-CD45RA^+$ (effector cells) (%) in healthy control, asthma, preterm and bronchopulmonary dysplasia (BPD) groups. Analysis with Mann–Whitney U-test.

B-cells, natural killer cells and natural killer T-cells/γδ T-cells

All study groups had low levels of B-cells without statistically significant differences between the groups (supplementary figure E2b). We found no statistically significant differences between the groups in the proportion of natural killer (NK) cells ($CD3^-CD56^+$; supplementary figure E2c) and the proportion of NKT-cells or γδ T-cells ($CD3^+CD56^+$; supplementary figure E2d) in BAL (supplementary table E5).

T-cell differentiation (CD27 and CD45RA)

The preterm group had lower percentages of naïve T-cells ($CD27^+CD45RA^+$; p=0.03) and central memory T-cells ($CD27^+CD45RA^-$; p=0.005; figure 3a and b), compared to the healthy control group, and a higher percentage of effector T-cells ($CD27^-CD45RA^+$; figure 3d), compared to the asthma group (p=0.02). Among the $CD3^+$ T-cells in BAL, effector memory cells ($CD27^-CD45RA^-$) were the dominant subset, followed by central memory cells ($CD27^+CD45RA^-$; data not shown).

Forkheadbox P3$^+$ (FoxP3$^+$) T-cells

The preterm and the BPD group had a lower proportion of $CD4^+FoxP3^+$T-cells compared to the asthma group (p=0.04, p=0.02, respectively; figure 4a). In addition, the pooled preterm-born group had fewer $CD4^+FoxP3^+$T-cells (p=0.02) compared to the asthma group. A lower percentage of $CD8^+FoxP3^+$T-cells in the preterm group compared both to the asthma and healthy control group was detected (p=0.03, p=0.04; figure 4b).

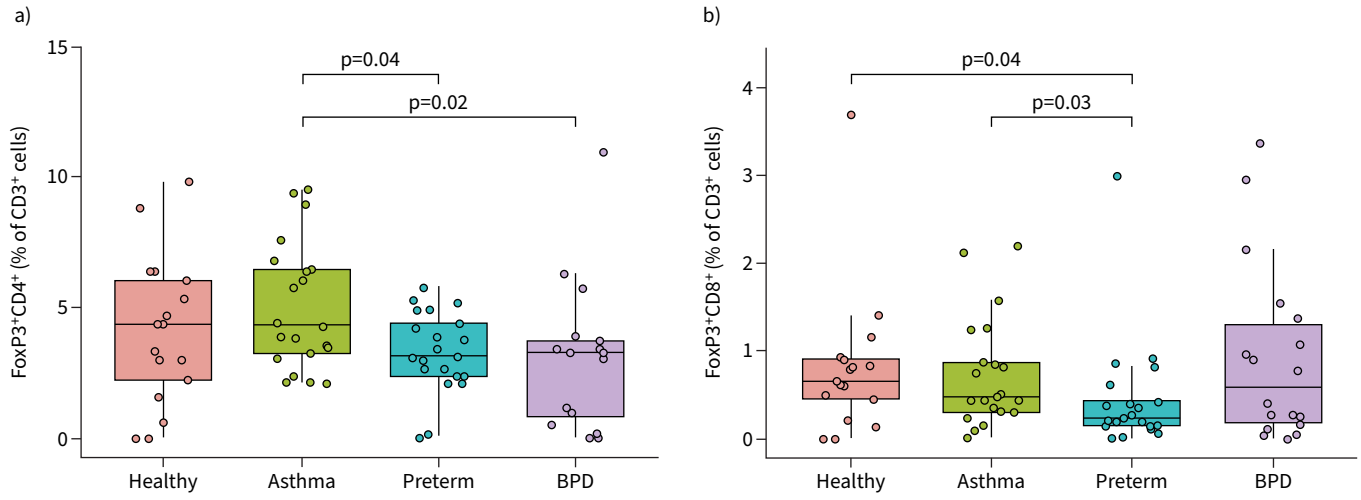

FIGURE 4 Dot plot showing the percentage of FoxP3$^+$ T-cells in bronchoalveolar lavage CD3$^+$ cells, with boxes indicating interquartile range, horizontal lines indicating the median and whiskers showing the 95% confidence interval. a) CD3$^+$CD4$^+$FoxP3$^+$ T-cells (%); b) CD3$^+$CD8+FoxP3$^+$ T-cells (%). Analysis with Mann–Whitney U-test. BPD: bronchopulmonary dysplasia.

Granzyme B and perforin in BAL fluid

To determine whether the elevated proportion of CD8$^+$ T-cells in BAL were accompanied by an altered concentration of CD8-specific markers of activity, granzyme B and perforin were analysed by ELISA in concentrated, cell-free BAL supernatants. Protein concentrations of granzyme B were below the limit of detection in 13 out of 24 subjects in the healthy control group, 13 out of 22 in the asthma group, 20 out of 21 in the preterm group and 19 out of 21 in the BPD group (figure 5a). Both the preterm and the BPD groups had significantly lower concentrations of granzyme B than the healthy control (p=0.04, p=0.04, respectively; figure 5a) and the asthma group (p=0.02, p=0.02, respectively; figure 5a). Detectable concentrations of perforin were found in samples from 85 out of 88 measured subjects. No statistical differences in perforin concentration were observed for the different study groups (figure 5b).

Correlations between T-cell subsets, soluble granzyme B, perforin and lung function

When the preterm and the BPD groups were pooled, we found that forced expiratory volume in 1 s (FEV_1) z-scores correlated with the percentage of CD4$^+$ BAL T-cells in a positive manner (figure 6a), and with CD8$^+$ BAL T-cells in a negative manner (r_s=0.38, p=0.03 and r_s= −0.44, p=0.009, respectively; figure 6b). Similar patterns were found when FEV_1/FVC ratio was used as a measure of airway obstruction (supplementary figure E3a and b). Moreover, positive correlations were observed between CD4/CD8 ratio and both FEV_1 and FEV_1/forced vital capacity (FVC) (figure 6c and supplementary figure E2c). We observed a positive correlation between reversibility of FEV_1 z-scores and both CD4$^+$ BAL T-cells (r_s=0.49, p=0.005) as well as CD4/CD8 ratio (r_s=0.46, p=0.008) in the pooled preterm group. Furthermore, there was a negative correlation between the reversibility of FEV_1 z-scores and CD8$^+$ BAL T-cells (r_s= −0.47, p=0.007; supplementary figure E4a–c). In line with these findings, there were also positive correlations between accumulated dose of PD20, CD4$^+$ BAL T-cells (r_s=0.47, p=0.02) and CD4/CD8 ratio (r_s=0.60, p=0.002), and negative correlation to CD8$^+$ BAL T-cells (r_s= −0.60, p=0.002) in the pooled preterm group (supplementary figure E4e and f). We observed no correlations between CD8$^+$ T-cells or CD3$^-$CD56$^+$ cells and levels of granzyme B (supplementary figure E5a and b). The levels of perforin demonstrated positive correlations to CD3$^-$CD56$^+$ cells (r_s=0.38, p=0.02), but not to CD8$^+$ T-cells (supplementary figure E5c and d).

Discussion

In the current study, we demonstrate that the number and proportions of leukocytes in BAL are similar in adults with former BPD compared to healthy control subjects and patients with asthma, despite an obstructive lung function impairment. More importantly, BPD subjects had a higher proportion of CD8$^+$ T-cells and a lower proportion of CD4$^+$ T-cells resulting, in a lower CD4/CD8 ratio compared to healthy controls. Furthermore, CD4$^+$ T-cells correlated in a positive manner, and CD8$^+$ T-cells in a negative manner, with expiratory flow rates. In addition, we show a higher proportion of activated T-cells in patients with BPD and differences in the proportions of CD8$^+$FoxP3$^+$ T-cells and markers of T-cell

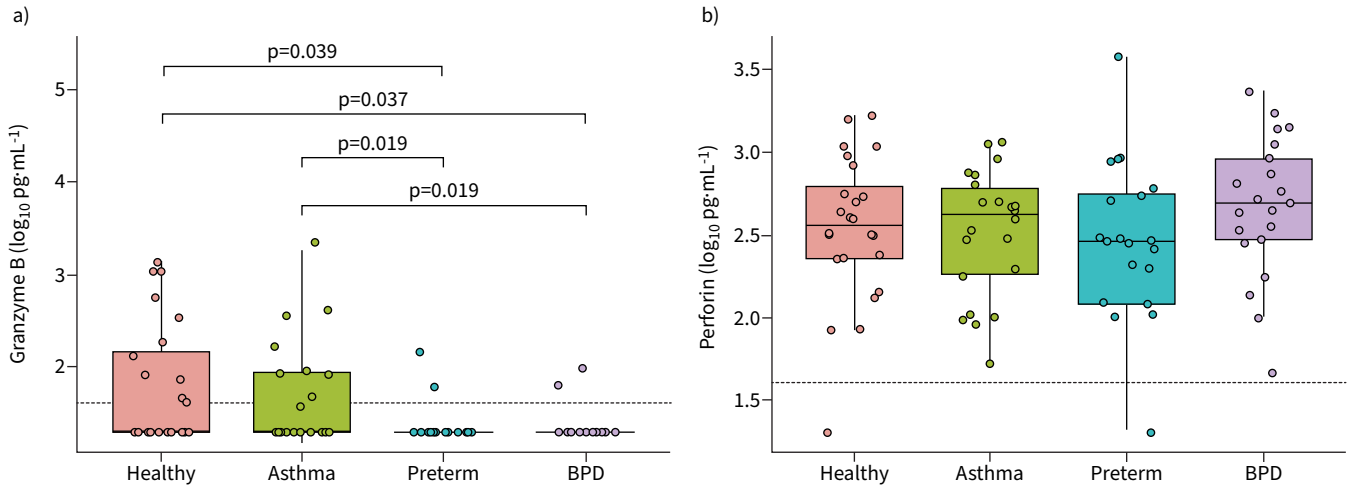

FIGURE 5 Protein concentrations of a) granzyme B and b) perforin were measured by ELISA in concentrated bronchoalveolar lavage (BAL) fluid. Dot plots show the distribution of detected levels of granzyme B and perforin, with boxes demonstrating interquartile range, horizontal line showing the median, and whiskers showing the 95% confidence interval. The dotted line represents a) the lowest level of quantitation assay range for granzyme B and b) the limit of detection for perforin. Analysis with Mann–Whitney U-test. BPD: bronchopulmonary dysplasia.

differentiation in the preterm group compared to healthy control subjects. Collectively, these findings indicate a mobilisation of $CD8^+$ T-cells in patients with former BPD, possibly leading to tissue remodelling and deterioration of lung function [31]. There is also evidence that preterm birth *per se* independently of BPD diagnosis could have an impact on the distribution of T-cells in the airways, as some of the findings were seen only in the preterm group, or in the pooled preterm group.

Recently, more focus on lung function trajectories and the importance of events before and after birth have emerged [32–34]. However, in this context, little is known about the mechanisms behind early lung function impairment and the development of chronic airway obstruction in adults.

Elevated proportions of $CD8^+$ T-cells and a lower CD4/CD8 ratio in patients with COPD implicate an altered inflammatory response as a result of an altered balance between $CD4^+$ and $CD8^+$ T-cells. Our findings suggest a similarity between inflammation in airway obstruction in nonsmoking adults with previous BPD and patients with COPD in terms of T-cell subsets in the lung.

In the BPD group, we found an increased proportion of $CD3^+CD8^+$ T-cells, suggesting that these T-cells increase the cytotoxic burden in the airways, since these cells can produce granzyme B, perforin and other cytotoxic agents [11]. This is in line with studies on mucosal biopsies from the large airways from three adolescents with severe BPD where a predominance of $CD8^+$ T-cell infiltrates were found [35]. The higher

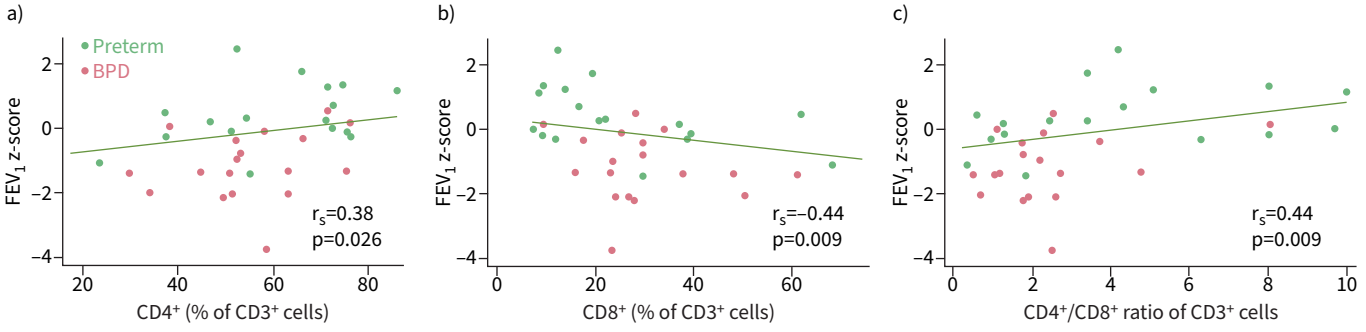

FIGURE 6 Correlation in pooled preterm group (preterm+bronchopulmonary dysplasia (BPD)) between a) $CD3^+CD4^+$ T-cells (%) in bronchoalveolar lavage (BAL) and post-bronchodilator forced expiratory volume in 1 s (FEV_1) z-scores; b) $CD3^+CD8^+$ T-cells (%) in BAL and post-bronchodilator FEV_1 z-scores; c) CD4/CD8 ratio of $CD3^+$ cells in BAL and post-bronchodilator FEV_1 z-scores. r_s: Spearman rank correlation coefficient.

burden of cytotoxic agents might lead to accelerated cell death of epithelial cells and increased cytokine production through activation of other cells [36]. Therefore, we measured concentrations of soluble granzyme B and perforin in cell-free BAL fluid. We detected lower concentrations of granzyme B in both BPD and preterm groups compared to the healthy and asthma group, while perforin did not markedly differ for any group. Hypothetically, the decreased concentrations of granzyme B might be due to increased degradation of granzyme B. Notably, previous studies have described neutralisation of granzyme B by target cells [37] and its inactivation of resident memory T-cells [38]. Moreover, there was no correlation between granzyme B and the proportion of $CD3^+CD8^+$ T-cells or NK cells, but we found a positive correlation between perforin and NK cells. In the BPD group, low levels of granzyme B might be explained by a higher proportion of $CD8^+$ cells that are resident memory T-cells. Altogether, the findings of decreased granzyme B and elevated $CD3^+CD8^+$ T-cells in the BPD group might implicate a different phenotype of obstructive lung disease than COPD and asthma. In BPD resident memory T-cells could play a greater role and the release of granzyme B downregulated for a protective effect thus maintaining a balance in the lung. Functional studies are warranted to further explore these immunological mechanisms in detail.

The transcription factor Forkhead box P3 (FoxP3) is the main regulator of T-regulatory (Treg) cell development and function, and it has been used as a marker for the identification of these cells [39–41]. Treg cells have been implicated in the pathogenesis of COPD and several autoimmune processes. The $CD4^+FoxP3^+$Treg subset is involved in the maintenance of immunological self-tolerance and immune homeostasis, possibly controlling inflammation to prevent tissue damage. Previous studies have shown that smokers, especially those with chronic bronchitis, have more $CD4^+FoxP3^+$ T-cells in BAL than never-smokers [42–44]. From a logical point of view, the decrease of $CD4^+$Tregs may also lead to a more aggressive inflammatory response in case of acute infection. Interestingly, both the preterm and the BPD group had lower proportions of $CD4^+FoxP3^+$ T-cells compared to the asthma group. This is in line with recent studies showing decreased BAL $CD4^+FoxP3^+$ T-cells in COPD patients [45, 46]. Other studies on COPD have demonstrated elevated levels of $CD4^+$Treg but with an altered regulatory function with a decrease in the expression of $FoxP3^+$ [47]. A prior study demonstrated lower $CD4^+$ T-cells and a slight decrease in $CD4^+FoxP3^+$ T-cells in cord blood from preterm-born infants with BPD compared to preterm-born infants without BPD [48], but a more recent study demonstrated increased $CD4^+FoxP3^+$ T-cells in peripheral blood preceding the development of BPD [49]. The authors demonstrated a decline over time in the levels of $CD4^+FoxP3^+$ T-cells in peripheral blood. Still, it is difficult to compare data from different compartments; cord blood *versus* peripheral blood *versus* BAL. In the preterm group, we found a lower proportion of $CD8^+FoxP3^+$ T-cells compared to the asthma group and healthy controls. The role of $CD8^+FoxP3^+$ T-cells in humans is less well described, but it has been demonstrated that these cells may be associated with pro-apoptotic effects in smokers with COPD [50], and have been claimed to mediate a suppressive function in prostate cancers [51]. The preterm group showed an altered profile in T-cell maturation with lower naïve T-cells and central memory T-cells compared to the healthy control group, and a higher percentage of effector T-cells compared to the asthma group. This could be of importance when considering pharmacological interventions like immunotherapy and vaccines [52–54]. The mechanisms behind the altered T-cell maturation remains unclear, but may be caused by an early inflammatory response due to the preterm birth in an immature immune system.

We also assessed markers of inflammation in peripheral blood, to explore signs of ongoing systemic inflammation. However, none of the groups displayed elevated levels of acute-phase proteins, suggesting that there was no systemic inflammation in either group.

We observed a skewed distribution of lymphocyte T-cell subsets in BAL fluid in patients with a history of BPD, and it related to lung function. Thus, we observed moderate positive correlations between both $CD4^+$ T-cells and CD4/CD8 ratio and measures of airways obstruction (FEV_1 and FEV_1/FVC) in the pooled group of preterm-born participants. In contrast, a moderate negative correlation was seen between $CD8^+$ T-cells and lung function. This finding further strengthens the potential tissue-damaging and cytotoxic effects of the $CD8^+$ T-cell subset and confirms earlier findings of negative correlation between $CD8^+$ T-cells from biopsies of the airways from individuals with smoking-induced chronic bronchitis and FEV_1 [55].

The strength of the study is a well-characterised study cohort with extensive data on neonatal conditions, demographics and lung function. In addition to the preterm-born participants, all with a history of extensive neonatal care, we also included patients with asthma and healthy control subjects. We included nonsmokers only, since it is known that current smoking *per se* affects the distribution of T-cell subsets in the airways [27]. The relatively small study groups constitute a limitation of the study. In addition, the preterm-born participants were born in 1992–1998, when the transition to modern neonatal care occurred;

therefore, relatively few study participants received surfactant and prenatal corticosteroids, which is now standard practice. This could have affected the outcome. However, to the best of our knowledge, the LUNAPRE cohort constitutes the largest BPD cohort investigated by bronchoscopy and we demonstrate clear-cut group differences for several key outcomes. Since it is difficult to find age-matched COPD subjects, we studied asthma as a disease with airway obstruction. Patients with former BPD are often treated as asthmatics as they share some of the symptoms and spirometry results [20, 32, 56, 57]. As BPD shares features of both COPD and asthma, but is most likely a unique disease, it merits the need for further phenotyping.

In conclusion, we report key findings from the first study investigating the inflammatory profile in BAL from young adults born preterm, with and without a history of BPD. This study advances evidence of an increased proportion of activated $CD8^+$ T-cells and a decreased proportion of $CD4^+$ T-cells in the peripheral airways in young adults with a history of BPD, indicating features of inflammation linked to COPD. The moderate correlations between T-cell parameters and lung function are compatible with the hypothesis that $CD8^+$ T-cells are involved in the mechanisms behind chronic airway obstruction in adults with a history of BPD.

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Data availability: Anonymised data will be shared upon reasonable request.

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Author contributions: C.M. Sköld conceived the study. P. Um-Bergström, E. Berggren Broström, A. Lindén, Å.M. Wheelock, B. Brundin, J. Gao, M. Pourbazargan, E. Melén and C.M. Sköld participated in the design and planning of the study. P. Um-Bergström, M. Pourbazargan and C.M. Sköld included study subjects and generated clinical data. C.M. Sköld performed bronchoscopies. P. Um-Bergström, M. Pourbazargan and E. Melén were responsible for lung function tests. B. Brundin and M. Ezerskyte handled BAL fluid. B. Brundin performed flow cytometric analyses. P. Um-Bergström carried out the data acquisition and analysis. C.M. Sköld, Å.M. Wheelock, A. Lindén, E. Melén and E. Berggren Broström provided financial support and essential infrastructure. P. Um-Bergström, A. Lindén, M. Ström, Å.M. Wheelock and C.M. Sköld interpreted data. P. Um-Bergström, J. Gao, M. Ström and Å.M. Wheelock provided statistical support and assistance in the interpretation of the results. P. Um-Bergström and C.M. Sköld drafted the manuscript. All authors were involved in the finalisation of the manuscript and approved the submitted version.

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