Modification of surface antigens in blood CD8+ T-lymphocytes in COPD: effects of smoking

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ABSTRACT: In contrast to the effects of cigarette smoke on T-lymphocyte subsets in the airways, it has not yet been determined whether smoking has immunomodulatory effects on surface antigens of peripheral blood T-lymphocytes and, if that is the case, whether these effects differ in smokers with and without chronic obstructive pulmonary disease (COPD).

The present authors have, therefore, examined the expression of the surface activation marker CD28, the levels of cytotoxic effector lymphocytes (CD27-/CD45RA+) and the expression of the lung type (Tc)1-specific chemokine receptor CXCR₃ on peripheral blood CD8+ T-lymphocytes. The present authors have also studied the chemotactic activity of CD8+ T-lymphocytes on monocyte chemotactic protein (MCP)-1 and compared 13 nonsmoking controls, 12 smokers with COPD and 14 smokers without airflow limitation.

There was a decrease in the total count of CD8+ T-cells and an increase in the CD4+/CD8+ ratio in smokers with COPD compared with smokers without COPD and controls. Expression of the Tc1-specific chemokine receptor CXCR₃ by CD8+ T-cells was increased in smokers with COPD compared with smokers without COPD and controls.

The expression of activated and of cytotoxic effector CD8+ T-cells in smokers with and without COPD showed an increase compared with controls. CD8+ T-cells from smokers with and without COPD showed a decrease in chemotactic activity to MCP-1 compared with controls.

In conclusion, chronic obstructive pulmonary disease may be a systemic immunomodulatory disease associated with the modification of surface antigens in blood CD8+ T-lymphocytes.

KEYWORDS: Chemokine receptor CXCR₃, chronic obstructive pulmonary disease, cigarette smoking, cytotoxic effector T-cells, peripheral blood CD8+ T-lymphocytes

Recent evidence suggests that lung type 1 (Tc1) CD8+ T-lymphocytes are implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD) [1]. However, there is little information regarding the role of peripheral blood CD8+ T-cells and the importance of their activation and cytotoxic/effector phenotype in this context.

Cigarette smoke is the main risk factor for developing COPD and exerts its primary effects on the lungs. However, there is clear evidence indicating the systemic effects of cigarette smoke, including an increase in the incidence of coronary disease, cancers of various organs and acute and chronic respiratory tract infections [2, 3]. Such effects may reflect cigarette smoke-induced impairment of the immune system [4] associated with T-cell anergy and modulatory effects on antigen-mediated signalling in T-lymphocytes

[5]. However, phenotype differences in circulating lymphocyte populations in smokers with and without COPD have not been directly compared.

Studies suggest that the proportion of peripheral blood CD8+ T-lymphocytes in smokers with COPD correlates significantly with the diffusing capacity of the lung for carbon monoxide (DL_{CO}). This results in a smaller proportion of CD8+ T-lymphocytes and a higher CD4+/CD8+ ratio in a COPD cohort with low DL_{CO} per unit of alveolar volume (DL_{CO}/VA) than in a COPD cohort with normal DL_{CO}/VA [6]. This suggests that peripheral blood T-lymphocyte abnormalities might be involved in the pathogenesis of airflow limitation. GLADER *et al.* [7] reported that cigarette smoking may induce a higher number of peripheral blood CD4+ T-cells than in subjects who have never smoked and smokers with COPD. Moreover, MAJORI *et al.* [8] examined Tc1/Tc2-cell

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cytokine profiles and reported a Tc1-like immune response of peripheral blood CD4⁺ T-cells with increased interferon (IFN)- γ expression in subjects with COPD. Interestingly, as well as producing the cytokines IFN- γ and interleukin-2, Tc1-cells also express the chemokine receptor CXCR₃ and its ligand CXCL10 during smoking-induced T-cell activation [1, 9]. CXCR₃ is one of the chemokine receptors induced predominantly on cytotoxic Tc1-cells [9]. Moreover, the release of CXCR₃-activating chemokines attracts Tc1-cells into the lungs, causing IFN- γ to induce more CXCR₃ ligands. This results in a self-perpetuating loop that may lead to accumulation of activated Tc1-cells in the peripheral lung [9]. Indeed, a study by SAETTA *et al.* [1] on smokers with COPD showed increased numbers of CXCR₃⁺ T-cells in both epithelium and submucosa of the airways compared with controls, indicating a Tc1-like inflammatory airway response in COPD. In contrast to the findings in lung parenchyma, LECKIE *et al.* [10] were unable to confirm this finding in induced sputum, where the percentage of CD8⁺ lymphocytes expressing CXCR₃ was lower than in blood from subjects with COPD. This indicates that different compartments should be analysed separately since induced sputum does not necessarily reflect the situation in lung parenchyma or in peripheral blood.

The prototypic co-stimulatory molecule CD28 is a receptor involved in the regulation of T-cell activation and in the generation of antigen-primed cells [11]. A substantial number of other co-stimulatory molecules and their ligands have been identified in recent years [12]. EKBERG-JANSSON *et al.* [12] reported a greater proportion of T-lymphocyte activation markers (human leukocyte antigen (HLA)-DR, CD26⁺, CD54⁺, CD69⁺) in bronchoalveolar lavage (BAL) fluid compared with blood in nonsmokers. Moreover, GLADER *et al.* [7] showed a clear correlation between expression of the activation marker CD69 on CD4⁺ T-cells and lung function (forced expiratory volume in one second (FEV₁)) in current smokers with and without COPD, indicating smoking-induced impairment of T-cell activation.

To promote the extravasation of peripheral blood lymphocytes, endothelial cells are known to secrete chemokines, such as monocyte chemotactic protein (MCP)-1, which guide lymphocytes to inflammatory sites or secondary lymphoid organs and into distinct compartments within the lung [13]. However, little is known about the modulatory effects of cigarette smoking on the expression of CXCR₃ and on the chemotactic activity of peripheral blood CD8⁺ T-lymphocytes to MCP-1 in this context.

Human CD8⁺ T-cells with a CD27⁺/CD45RA⁻ phenotype are memory cells of low cytotoxicity, while those with a CD27⁻/CD45RA⁺ phenotype are cytotoxic effector cells [14]. An increased infiltration of cytotoxic CD8⁺ T-lymphocytes into the central airways [15] and the lung parenchyma [16] and increased numbers of these cells in sputum samples [17] have been reported in COPD patients.

Therefore, in the present study, the authors examined the potential role of cigarette smoking in modulating the surface activation antigen CD28, the numbers of cytotoxic effector T-cells (CD27⁻/CD45RA⁺) and the expression of the Tc1-specific chemokine receptor CXCR₃ surface antigen on peripheral

blood CD8⁺ T-lymphocytes. The chemotactic activity of CD8⁺ T-cells to MCP-1 was also studied and nonsmokers were compared with smokers with and without COPD.

MATERIALS AND METHODS

Subjects

The study population consisted of 13 healthy nonsmokers, 14 current smokers without respiratory symptoms or airflow limitation and 12 current smokers with respiratory symptoms and moderate to severe airflow limitation [18] (table 1).

None of the subjects were using oral or inhaled corticosteroids, receiving immunosuppressive treatment, reported any other serious illness or acute viral disease during the 2 months preceding the test or had tuberculosis, parasite infections, history of allergies or asthma. The nonsmokers and smokers (≥ 10 pack-yr and ≤ 40 pack-yr) with normal chest radiographic and spirometry findings were selected from a population that had undergone an annual health check-up. COPD was diagnosed according to the criteria recommended by the National Institutes of Health/World Health Organization workshop summary [19]. The smokers with COPD (≥ 10 pack-yr) had a history of cough with sputum production and/or dyspnoea on most days of the month for ≥ 3 months a year during > 2 yrs before the study. They also presented airflow limitation on spirometry (FEV₁/forced vital capacity $< 70\%$ and FEV₁ $< 80\%$ of predicted value), as defined by the Global Initiative for Chronic Obstructive Lung Disease initiative [18, 19]. The airflow limitation in these patients was irreversible, as shown by a negative immediate response to inhalation of 200 μ g of albuterol ($\leq 12\%$ reversibility). Their pulmonary function had been stable for several months under observation. There was no statistical difference in cigarette smoke exposure and age between smokers with and without COPD. The study was approved by the ethics committee of the University of Cologne, Cologne, Germany.

TABLE 1 Characteristics of healthy nonsmokers, smokers and subjects with chronic obstructive pulmonary disease (COPD)

	Nonsmokers	Smokers	Smokers with COPD
Subjects n	13	14	12
Age yrs	48.3 $\pm$ 2.7	47.4 $\pm$ 4.3	56.0 $\pm$ 2.1
Male:female	7:6	8:6	7:5
FEV₁ % pred	100.6 $\pm$ 3.1	104.5 $\pm$ 3.6	46.7 $\pm$ 3.9 [#]
FEV₁/FVC % pred	84.3 $\pm$ 1.5	84.7 $\pm$ 2.2	54.3 $\pm$ 2.7 [#]
FVC % pred	100.9 $\pm$ 2.8	106.1 $\pm$ 4.5	68.8 $\pm$ 4.4 [#]
RV/TLC % pred	28.9 $\pm$ 1.8	33.6 $\pm$ 3.4	66.4 $\pm$ 2.8 [#]
RV % pred	110.0 $\pm$ 7.0	118.6 $\pm$ 11.1	211.5 $\pm$ 8.3 [#]
IC % pred	86.6 $\pm$ 3.2	89.4 $\pm$ 7.4	60.7 $\pm$ 6.0 [#]
Pack-yr		33.8 $\pm$ 0.5	35.3 $\pm$ 2.8

Data are presented as mean $\pm$ SD, unless otherwise stated. FEV₁: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; IC: inspiratory capacity.

[#]: p < 0.01 versus smokers and nonsmokers using the Neumann-Keuls test.

Pulmonary function study

Spirometric parameters and lung volumes were measured according to the recommendations of the American Thoracic Society [20] using a body plethysmograph (Masterlab; Viasys, Würzburg, Germany) and were expressed as a percentage of predicted using the prediction formula of GOLDMAN and BECKLAGE [21] (table 1).

Isolation of CD8⁺ T-lymphocytes from peripheral blood

Peripheral venous blood from all subjects was drawn after obtaining informed consent. Aliquots were submitted to the laboratory for differential white blood cell count (table 2). Peripheral blood mononuclear cells were separated from acid citrate dextrose venous blood and obtained after Ficoll-Paque gradient centrifugation. T-cells were isolated from mononuclear cells by immunomagnetic depletion of CD14, CD16, CD56 and HLA Class II DR/DP (B-cells, natural killer-cells, monocytes, granulocytes) with a T-cell isolation kit (DynaL Biotech, Hamburg, Germany) by negative selection. The selected cells were routinely $\geq 95\%$ CD3⁺. To obtain purified CD8⁺ T-cells, the present authors used the CD8⁺ isolation kit (DynaL Biotech) for rapid immunomagnetic separation of CD4⁺ T-cells and CD8⁺ T-lymphocytes according to the manufacturer's instructions. The purity of the CD8⁺ T-cells determined by flow cytometry was routinely $\geq 98\%$.

CD8⁺ T-cell chemotaxis assay

The chemotaxis assay was performed using a 96-well microchamber, as previously described elsewhere [22]. The chemoattractant MCP-1 was diluted in RPMI 1640 medium containing 1% foetal calf serum (FCS) to final concentrations

of $0.125 \mu\text{g}\cdot\text{mL}^{-1}$ and $0.25 \mu\text{g}\cdot\text{mL}^{-1}$. Then, $50 \mu\text{L}$ were placed in the lower chamber. Purified CD8⁺ T-cells were resuspended at a concentration of $5\cdot 10^6 \text{ cells}\cdot\text{mL}^{-1}$ in RPMI / 1% FCS and incubated for 1 h at 37°C in an atmosphere containing 5% CO_2 . A cell suspension of $50 \mu\text{L}$ ($2.5\cdot 10^5 \text{ cells}$) was added to the upper part of the chamber separated from the lower chamber by a $5\text{-}\mu\text{m}$ -pore-size polycarbonate, polyvinylpyrrolidone-free filter (Applichem, Darmstadt, Germany) coated with mouse type IV collagen (BD Biosciences, Bedford, England). The Boyden chamber was incubated for 120 min at 37°C in an atmosphere containing 5% CO_2 . The filters were carefully removed, fixed in 70% methanol and stained with Diff-Quick (Dade Behring, Newark, NJ, USA). After removal of non-migrated cells from the upper surface, migrated and adhered cells on the lower surface of the membrane were counted by measuring their area with a defined phase contrast microscope (Phase Contrast ELWD 0.3 (OD 75 mm); Nikon, Cambridge, MA, USA) with a 10×10 field diagram. The number in each field was calculated. CD8⁺ T-lymphocyte migration was quantified by counting the total number of cell migrations completely through the filter in duplicate samples. The number of migrated cells in the negative control, which reflects spontaneous migration, was subtracted from the number of migrated cells in the samples.

Four-colour fluorescence-activated cell sorting analysis and gating strategy

Whole blood ($100 \mu\text{L}\cdot\text{tube}^{-1}$) was incubated with CXCR₃+conjugated fluorescein-isothiocyanate (FITC)-Antibody (Ab) (R&D Systems, Wiesbaden, Germany), CD4+conjugated FITC-Ab, CD45RA+conjugated FITC-Ab, CD28+conjugated PE-Ab, CD27+conjugated phycoerythrin (PE)-Ab, CD3+conjugated phycoerythrin and texas red (ECD)-Ab or CD8+conjugated phycoerythrin and cyanin (PC)5-Ab (Beckman Coulter, Krefeld, Germany) for 20 min in darkness at room temperature according to the study protocol. FITC-, PE- and PC5-conjugated isotype-immunoglobulin (Ig)G control and isotype-IgG-ECD control were purchased from Beckman Coulter. The red blood cells were lysed using lysing solution (Beckman Coulter) for 20 min. The samples were then vortexed and centrifuged for 5 min at $415\times g$ and 4°C . The supernatant was discarded and the pellet resuspended in 1 mL PBS. A total of 50,000 cells were analysed by flow cytometry in each test. Cells were initially gated on the basis of forward and side scatter characteristics, with gates set to remove debris and platelets. Results were expressed as the percentage of cells exhibiting positive fluorescence.

Data analysis

Data are reported as mean $\pm$ SEM. A Kruskal-Wallis one-way ANOVA was used to evaluate significant differences between groups and, when significance was found, *post hoc* between-group analysis was performed with the Newman-Keuls test. A p-value <0.05 was considered significant.

RESULTS

Peripheral blood cell counts

As table 2 shows, there was a significant increase in peripheral blood neutrophils (expressed as absolute neutrophil counts and as percentage of all leukocytes) in smokers with COPD compared with nonsmokers ($p<0.05$). There was a decrease in

TABLE 2

Peripheral blood cell counts in nonsmokers, smokers and subjects with chronic obstructive pulmonary disease (COPD)

	Nonsmokers	Smokers	Smokers with COPD
Subjects n	13	14	12
Leukocytes			
$10^3\cdot\mu\text{L}^{-1}$	6.5 ± 0.3	7.8 ± 0.8	8.8 ± 0.7
Lymphocytes			
$10^3\cdot\mu\text{L}^{-1}$	2.0 ± 0.1	2.3 ± 0.3	1.9 ± 0.1
Leukocytes %	31.3 ± 2.3	29.7 ± 1.5	$22.9\pm 1.5^\#$
Eosinophils			
$10^3\cdot\mu\text{L}^{-1}$	0.16 ± 0.03	0.15 ± 0.02	0.24 ± 0.05
Leukocytes %	2.47 ± 0.4	2.01 ± 0.2	2.85 ± 0.4
Neutrophils			
$10^3\cdot\mu\text{L}^{-1}$	3.79 ± 0.3	4.67 ± 0.5	$5.79\pm 0.5^\ddagger$
Leukocytes %	57.5 ± 26	59.9 ± 1.9	$65.2\pm 1.7^\ddagger$
Monocytes			
$10^3\cdot\mu\text{L}^{-1}$	0.4 ± 0.03	0.4 ± 0.06	0.5 ± 0.06
Leukocytes %	5.63 ± 0.36	5.16 ± 0.50	6.12 ± 0.46
Basophils			
$10^3\cdot\mu\text{L}^{-1}$	0.05 ± 0.007	0.06 ± 0.009	0.07 ± 0.015
Leukocytes %	0.90 ± 0.12	0.92 ± 0.13	0.92 ± 0.13

Data are presented as mean $\pm$ SD, unless otherwise stated. #: $p<0.01$ versus smokers and nonsmokers using the Newman-Keuls test; ‡ : $p<0.05$ versus nonsmokers using the Newman-Keuls test.

FIGURE 1. Individual values for CD4+/CD8+ ratios of peripheral blood T-lymphocytes. The data presented are derived from separate experiments using cells from 13 nonsmokers, 14 smokers without chronic obstructive pulmonary disease (COPD) and 12 smokers with COPD. There was a significant increase in CD4+/CD8+ ratio in smokers with COPD compared with smokers without COPD and nonsmokers, while no difference was found between smokers without COPD and nonsmokers. Median values are represented by —. *: $p < 0.05$; **: $p < 0.01$.

total lymphocytes in smokers with COPD compared with smokers without COPD and controls, which was statistically significant when expressed as a percentage of all leukocytes ($p < 0.01$) but not as absolute lymphocyte counts.

Total count and ratio of CD4+ and CD8+ T-lymphocytes in peripheral blood

There was a significant increase in the percentage of CD4+/CD3+ T-cells and a significant decrease in the percentage of CD8+/CD3+ T-lymphocytes in smokers with COPD (CD4+: $73.4 \pm 2.1\%$, $p < 0.01$; CD8+: 22.7 ± 1.6 ; $p < 0.01$) compared with nonsmokers (CD4+: $60.5 \pm 2.0\%$, CD8+: 34.2 ± 2.0) and smokers without COPD (CD4+: $65.9 \pm 2.5\%$, $p < 0.05$; CD8+: 29.8 ± 2.1 ; $p < 0.05$; graph not shown). As figure 1 shows, these smoking-independent changes were also found in the CD4+/CD8+ ratio in smokers with COPD (3.5 ± 0.4) compared with smokers without COPD (2.4 ± 0.3 ; $p < 0.05$) and nonsmokers (1.9 ± 0.2 ; $p < 0.01$).

Th/Th2 differentiation of CD8+ T-lymphocytes in peripheral blood

Figures 2a, b and c show original flow cytometry analyses based on PC-5-conjugated CD8+ T-lymphocytes and FITC-conjugated CXCR3+ T-cell populations from a nonsmoker and a smoker without COPD and from a smoker with COPD. Figure 2d summarises the data of parallel stainings for ECD-conjugated CD3+ T-cells and PC5-conjugated CD8+ and FITC-conjugated CXCR3+ dot blots and shows flow cytometry analysis results of CXCR3+ peripheral blood T-lymphocytes expressed as a percentage of total CD8+ T-cells. All events above the isotype control were defined as CXCR3+.

There was a significant increase in the percentage of CXCR3+/CD8+ T-lymphocytes in smokers with COPD ($70.1 \pm 1.8\%$) compared with nonsmokers ($60.7 \pm 2.5\%$; $p < 0.05$) and smokers without COPD ($60.9 \pm 2.6\%$; $p < 0.05$). In contrast, there was no statistical difference between CXCR3+/CD8+ T-lymphocytes in

smokers without COPD and nonsmokers ($p > 0.05$, nonsignificant (NS)). This indicates that the increased number of CXCR3+/CD8+ T-cells in COPD is smoking-independent (fig. 2d). The percentage of positive cells in these subsets stained with mouse IgG mAb was $< 1\%$.

Activation of CD8+ T-lymphocytes in peripheral blood

CD28 is a co-stimulatory receptor involved in the regulation of T-cell activation [11]. Staining for ECD-conjugated CD3+ T-cells, PC5-conjugated CD8+ and PE-conjugated CD28+ was performed on dot blots from a nonsmoker, a smoking individual and from a subject with COPD, as shown in figures 3a, b and c. Summarised individual values of flow cytometry results of CD28+ peripheral blood T-lymphocytes were expressed as percentages of total CD8+ T-cells. As figure 3d shows, there was a significant increase in the percentage of CD28+/CD8+ T-cells in smokers without COPD ($72.9 \pm 3.7\%$; $p < 0.05$) and in subjects with COPD ($75.4 \pm 3.9\%$; $p < 0.05$) compared with nonsmokers ($61.0 \pm 2.7\%$). In contrast, there was no significant difference between CD28+/CD8+ T-lymphocytes in smokers with and without COPD ($p > 0.05$, NS). This indicates that the activation of CD8+ T-cells in both groups could be smoking-related. The percentage of positive cells in these subsets stained with mouse IgG mAb was $< 1\%$.

Chemotactic activity of CD8+ T-lymphocytes to MCP-1

There was no significant difference in spontaneous migration of peripheral blood CD8+ T-lymphocytes between groups (smokers: 1.58 ± 0.23 ; COPD: 1.89 ± 0.54 ; nonsmokers: 1.79 ± 0.15 ; NS for all).

However, as figure 4 shows, there was decreased chemotactic activity of CD8+ T-lymphocytes to MCP-1 at a concentration of $0.125 \mu\text{g}\cdot\text{mL}^{-1}$ in smokers without COPD (6.3 ± 0.8) and in smokers with COPD (6.7 ± 0.7) compared with nonsmokers (9.9 ± 1.0 ; $p < 0.05$). There was no statistical difference between smokers with and without COPD indicating a smoking-dependent decreased chemotactic activity of CD8+ T-cells to MCP-1.

At the higher concentration of $0.25 \mu\text{g}\cdot\text{mL}^{-1}$, the migration of peripheral blood CD8+ T-lymphocytes to MCP-1 was not statistically different between groups: smokers without COPD (7.1 ± 1.0) and smokers with COPD (8.8 ± 0.8) compared with nonsmokers (10.7 ± 1.4 ; $p > 0.05$).

Cytotoxic/effector phenotype of peripheral blood CD8+ T-lymphocytes

In the present study the authors attempted to clarify smoking-induced immunomodulatory effects on cytotoxic effector CD8+ T-cells (CD27-/CD45RA+) using a parallel colour flow cytometric analysis, including PC5-conjugated CD8+, FITC-conjugated CD45RA+ and PE-conjugated CD27+ dot blots (figs. 5a, b and c). In figure 5d, CD27-/CD45RA+ peripheral blood CD8+ T-lymphocytes are expressed as the percentage of total CD8+ T-cells from individual patients. The data presented are derived from separate experiments using cells from 13 nonsmokers, 14 smokers without and 12 smokers with COPD. There was a significant increase in the percentage of CD27-/CD45RA+ T-lymphocytes in relation to total CD8+ T-cells from smokers without COPD ($18.4 \pm 2.4\%$) and from smokers with COPD (23.5 ± 4.3) compared with nonsmokers (8.4 ± 1.2 ;

FIGURE 2. a–c) Representative original fluorescence-activated cell sorting (FACS) analyses of ECD-conjugated CD3+ T-cells and PC-5-conjugated CD8+ and FITC-conjugated CXCR3+ populations of a nonsmoking individual (a; CD3+/CD8+ 57.5%), a smoking individual (b; CD3+/CD8+ 58.4%) and a subject with chronic obstructive pulmonary disease (COPD) (c; CD3+/CD8+ 70.2%). d) Individual results of FACS analysis of ECD-conjugated CD3+ T-cells and PC-5-conjugated CD8+ and FITC-conjugated CXCR3+ dot plots of 13 nonsmokers, 14 smokers without and 12 smokers with COPD. There was a significant increase in the percentage of CXCR3+ T-lymphocytes in relation to total CD8+ T-cells in smokers with COPD compared with nonsmokers and smokers without COPD. In contrast, there was no significant difference between COPD and nonsmokers. Median values are represented by —. *: $p < 0.05$.

$p < 0.05$ versus smokers; $p < 0.01$ versus COPD). This indicates a smoking-related increase in cytotoxicity of CD8+ T-cells. The percentage of positive cells in these subsets stained with mouse IgG mAb was <1%.

DISCUSSION

In the present study the authors found a higher number of CD4+ T-cells, a lower number of CD8+ T-lymphocytes and a higher CD4+/CD8+ ratio in the peripheral blood of smokers with COPD compared with smokers without airflow limitation and nonsmokers (fig. 1). As table 2 shows, there was no difference in total lymphocyte counts expressed in absolute values between all groups. This compartment-specific decrease in CD8+ T-lymphocytes is in contrast to previous findings of increased CD8+ T-cell populations in lung parenchyma [23], pulmonary arteries [23], peripheral airways [24] and induced sputum [25] of smokers with COPD. This was underlined by the fact that the CD4+/CD8+ ratio was significantly lower in induced sputum from smokers with COPD compared with smokers without COPD and nonsmokers [25]. However, a parallel T-cell subset analysis in the airways and in peripheral

blood from smokers with and without COPD is needed to clarify this point. The smoking-independent results of the present study in COPD may be consistent with a study by AMADORI *et al.* [26], who presented evidence explaining why smokers may develop COPD and suggested that individuals with a genetically determined increase in the CD8+ population might be more susceptible to further CD8+ T-cell abnormalities [27]. This increased susceptibility may in turn be enhanced by cigarette smoking [15] or repeated virus infections [28], inducing an abnormal inflammatory response contributing to the pathogenesis of COPD. In contrast to the present authors' results, de JONG *et al.* [29] found a lower CD4+/CD8+ ratio in peripheral blood from COPD patients compared with non-smoking healthy control subjects. However, in that particular study all COPD patients were nonsmokers [29]. KIM *et al.* [6] studied subgroups of smokers with COPD and reported that a reduced DL_{CO}/VA was correlated with a lower proportion of peripheral blood CD8+ T-lymphocytes and a higher CD4+/CD8+ ratio compared to COPD patients with a normal DL_{CO}/VA . This suggests that T-lymphocyte abnormalities might be involved in the pathogenesis of airflow limitation.

FIGURE 3. a-c) Representative original fluorescence-activated cell sorting (FACS) analyses based on ECD-conjugated CD3+/PC5-conjugated CD8+ and PE-conjugated CD28+ populations of a nonsmoker (a) and smokers without (b) and with chronic obstructive pulmonary disease (COPD; c). d) CD28+ peripheral blood T-lymphocytes expressed as a percentage of CD8+ T-cells of 13 nonsmokers, 14 smokers without and 12 smokers with COPD. There was a significant increase in the percentage of CD28+ T-lymphocytes in smokers with and without COPD compared with nonsmokers, while no difference was found between smokers with and without COPD. Median values are represented by —. *: $p < 0.05$.

FIGURE 4. CD8+ T-cell chemotaxis assay of monocyte chemotactic protein (MCP)-1 ($0.125 \mu\text{g}\cdot\text{mL}^{-1}$) stimulated peripheral blood T-lymphocytes expressed as CD8+ T-lymphocyte migration/field using cells from 13 nonsmokers, 14 smokers without and 12 smokers with chronic obstructive pulmonary disease (COPD). There was a significant decreased chemotactic activity of CD8+ T-lymphocytes to MCP-1 from smokers with and without COPD compared with nonsmokers but no statistical difference between smokers with and without COPD. Median values are represented by —. *: $p < 0.05$.

Recent evidence suggests that CD8+ T-cells implicated in COPD are of the Tc1 phenotype [30]. Indeed, in the present study the authors were able to demonstrate a smoking-independent increase in the Tc1-specific CXCR₃+ surface antigen of peripheral blood CD8+ T-lymphocytes in smokers with COPD compared with smokers without airflow limitation (fig. 2). The release of CXCR₃-activating chemokines attracts Tc1-cells into the lungs, followed by expression of IFN- γ , which may lead to accumulation of activated Tc1-cells in the peripheral lung [30]. The specificity of this effect was confirmed by SAETTA *et al.* [1], who found increased numbers of CXCR₃+ T-cells in both epithelium and submucosa of the airways in smokers with COPD compared with controls. Interestingly, TZANAKIS *et al.* [25] noted a decrease in IFN- γ -producing CD8+ T-cells (Tc1) in sputum from smokers with COPD compared with smokers and nonsmokers. This is consistent with the findings of LECKIE *et al.* [10], who found a lower percentage of CD8+ T-lymphocytes expressing CXCR₃+ in induced sputum from COPD patients compared with nonsmokers. This indicates that sputum results do not always reflect changes of T-cell phenotype seen in the airways and in peripheral blood. XIE *et al.* [9] demonstrated that CXCR₃ played a critical role in T-cell transmigration to sites of inflammation.

FIGURE 5. a-c) Representative original fluorescence-activated cell-sorting (FACS) analyses of PC5-conjugated CD8+ and PE-conjugated CD27+ T-cells and FITC-conjugated CD45RA+ T-cell population from a nonsmoker (a), a smoker without (b) and a smoker with chronic obstructive pulmonary disease (COPD; c). d) Summary of the individual values of parallel colour stainings of CD27- and CD45RA+ peripheral blood T-lymphocytes expressed as a percentage of CD8+ T-cells from 13 nonsmokers, 14 smokers without and 12 smokers with COPD. There was a significant increase in the percentage of CD27-/CD45RA+ T-lymphocytes in relation to total CD8+ T-cells of smokers with and without COPD compared with nonsmokers. In contrast, there was no statistical difference between CD27-/CD45RA+ T-lymphocytes in relation to CD8+ T-cells in smokers with and without COPD. Median values are represented by —. *: $p < 0.05$; **: $p < 0.01$.

These findings provide further evidence that dysregulation of Tc1-agonist chemokines and their cognate receptors, such as CXCR₃, might contribute to the immunopathology of COPD [31] in a smoking-independent manner.

Previous studies have not comprehensively analysed different activation markers on T-cells distinguishing between expression on CD4+ and CD8+ T-cells. Recently, GLADER *et al.* [7] demonstrated that the expression of the surface activation marker CD69 on CD4+ T-cells in smokers and COPD patients correlated with lung function measured as FEV₁ % pred, indicating a protective effect for smokers. Previous studies reported that no differences were found in CD25+ [8], CD69+ [10] and CD45RO+ [29] surface activation marker expression of T-cells in peripheral blood of COPD patients and of healthy subjects. EKBERG-JANSSON *et al.* [32] presented lower numbers of CD8+ T-cell activation markers (CD57+ and CD28+) in BAL fluid of smokers compared with nonsmokers. In comparison to previous studies in the airways, the present authors demonstrated that cigarette smoking increased the expression of the

surface activation marker CD28 on CD8+ T-lymphocytes in peripheral blood of smokers with and without airway obstruction compared with nonsmokers (fig. 3). Where the systemic activation levels reflect a general capability to mount a higher protective T-cell response to airway infections, although these COPD-patients might be coping better with repeated infections, accumulation of activated CD8+ T-cells in peripheral blood may be followed by a lower number of CD28+ CD8+ T-cells in the airways [30]. This could be of importance for disease progression. Indeed, recurring exacerbations induced by airway infections have been implicated in declining lung function in COPD patients [33].

The present authors also investigated the systemic immunotoxicity of cigarette smoking as measured by the cytotoxic effector (CD27-/CD45RA+) peripheral blood CD8+ T-lymphocytes. It was found that cigarette smoking increased the percentage of cytotoxic effector CD8+ T-lymphocytes in peripheral blood of smokers and COPD patients compared with nonsmokers (fig. 5). This subpopulation of CD8+

T-lymphocytes causes lysis of target cells *via* two mechanisms: 1) membranolysis, in which secreted molecules, such as perforin and granzymes, form pores in the membrane of target cells [17]; and 2) apoptosis, mediated by the triggering of apoptosis-inducing (Fas-like) surface molecules of the target cells [17, 34, 35]. Moreover, cigarette smoke reduced chemotactic activity of peripheral blood CD8⁺ T-cells to MCP-1 at a concentration of 0.125 $\mu\text{g}\cdot\text{mL}^{-1}$ (fig. 4), but did not change the spontaneous migration of CD8⁺ T-lymphocytes of smokers with and without COPD compared with nonsmokers. Interestingly, these smoking-induced significant changes in chemotactic activity to MCP-1 could not be found at a higher concentration of 0.25 $\mu\text{g}\cdot\text{mL}^{-1}$. This indicates that the response of T-cells to MCP-1 is dose-dependent [13]. The observation that cigarette smoke reduced the chemotactic activity but induced a higher cytotoxicity and activation of peripheral blood CD8⁺ T-lymphocytes underscores the thesis that cigarette smoke induces the extravasation predominantly of memory, rather than cytotoxic CD8⁺ T-cells, in order to prolong cytotoxic effector CD8⁺ T-cell effects in local compartments [13].

In conclusion, the present findings demonstrate that surface antigen modification on peripheral blood CD8⁺ T-lymphocytes plays an important role in the pathogenesis of chronic obstructive pulmonary disease and that this may be generated only in part by cigarette smoking. The scientific significance and validity of the present results may be influenced by small sample sizes, age, smoking history and sex in each group. These effects could potentially affect the clinical relevance of the present findings and should, therefore, be addressed in future studies.

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