ERJ Express. Published on October 18, 2006 as doi: 10.1183/09031936.00133205

Modification of Surface Antigens in Blood CD8$^+$-T-Lymphocytes in COPD – Effects of Smoking

Andrea Koch, MD[1], Maciej Gaczkowski, MD[1], Graham Sturton, PhD[3], Peter Staib, MD[2], Timo Schinköthe, PhD[2], Elfriede Klein PhD[1], Andrea Rubbert MD[2], Kevin Bacon, PhD[4], Klaus Waßermann Prof, MD[1], Erland Erdmann, Prof, MD[1]

[1]University of Cologne, Clinic III for Internal Medicine, Department of Pneumology, Cologne, Germany; [2]University of Cologne, Clinic I for Internal Medicine, Department of Haematology, Cologne, Germany; [3]Thoracic Medicine, Imperial College at National Heart & Lung Institute, London, UK, [4]Bayer Ltd, Stoke Court, Berkshire, UK

Address:

Corresponding author:

Dr. Andrea Koch

University of Cologne

Medical Clinic III

Department of Pneumology

Joseph-Stelzmann-Str. 9

50924 Köln (Cologne)

Germany

Telephone: +49 221 478 3793

Fax: +49 221 478 3137

e-mail: andrea.koch@uni-koeln.de

Running title: Smoking and surface antigens of T-cells

ABSTRACT

In contrast to the effects of cigarette smoke on T-lymphocyte subsets in the airways, we still do not know whether smoking has immunomodulatory effects on surface antigens of peripheral blood T-lymphocytes and if so, whether these effects differ in smokers with and without COPD.

We therefore examined the expression of the surface activation marker CD28, the numbers of cytotoxic effector lymphocytes ($CD27^-/CD45RA^+$) and expression of the Tc1-specific chemokine receptor $CXCR_3^+$ on peripheral blood $CD8^+$-T-lymphocytes using parallel flow cytometry. We also studied chemotactic activity of $CD8^+$-T-lymphocytes to MCP-1 using a Boyden chamber method and compared 13 non-smoking 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 to smokers without COPD and controls. Expression of the Tc1-specific chemokine receptor $CXCR_3^+$ by $CD8^+$ T-cells was increased in smokers with COPD compared to smokers without COPD and controls.

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

In conclusion COPD may be a systemic immunomodulatory disease associated with modification of surface antigens in blood $CD8^+$-T-lymphocytes.

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KEY WORDS

Peripheral blood $CD8^+$ -T-lymphocytes; cigarette-smoking, COPD, cytotoxic effector T-cells, $CXCR_3$

ABBREVIATIONS

COPD, chronic obstructive pulmonary disease; MCP-1, macrophage chemoattractant protein-1; Ag, antigen; FEV_1, forced expiratory volume in 1 sec; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity, IC, inspiratory capacity.

INTRODUCTION

Recent evidence suggests that lung type 1 (Tc1) $CD8^+$ T-lymphocytes are implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD) [1] but 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 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 signaling 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 (DLCO) resulting in a smaller proportion of $CD8^+$-T-lymphocytes and a higher $CD4^+/CD8^+$ ratio in COPD with low DLCO/VA than in a COPD cohort with normal DLCO/VA [6]. This suggests that peripheral blood T-lymphocyte abnormalities might be involved in the pathogenesis of airflow limitation. Glader et al., reported that cigarette smoking may induce a higher number of peripheral blood $CD4^+$-T-cells than in both subjects who have never smoked and smokers with COPD [7]. Moreover, Majori and colleagues [8] examined Tc1/Tc2-cell cytokine profiles and reported a Tc1-like immune response of peripheral blood $CD4^+$ T-cells with increased IFNγ expression in subjects with COPD. Interestingly, as well as producing the cytokines IFNγ and IL-2, Tc1 cells also express the chemokine receptor $CXCR_3$ and

its ligand CXCL10 during smoking-induced T cell activation [1, 9]. $CXCR_3$ is one of the chemokine receptors induced predominantly on cytotoxic Tc1 cells [9]. Moreover, release of $CXCR_3$ -activating chemokines attracts Tc1 cells into the lungs causing IFNγ to induce more $CXCR_3$ 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 $CXCR_3^+$ T-cells in both epithelium and submucosa of the airways compared with controls, indicating a Tc1-like inflammatory airway response in COPD. But in contrast to the findings in lung parenchyma, Leckie and coworkers were not able to confirm this finding in induced sputum, where the percentage of $CD8^+$ lymphocytes expressing $CXCR_3$ was lower than in blood from subjects with COPD [10. 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] but a substantial number of other co-stimulatory molecules and their ligands have been identified during the past years [12]. Ekberg-Jansson and coworkers reported [12] a greater proportion of T-lymphocyte activation markers (HLA-DR, $CD26^+$, $CD54^+$, $CD69^+$) in bronchoalveolar lavage (BAL) fluid compared to blood in non-smokers. Moreover, Glader et al. [7] showed a clear correlation between expression of the activation marker CD69 on $CD4^+$ T-cells and lung function (FEV_1) 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-1 (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 $CXCR_3$ 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]. 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 reorted in COPD patients.

Therefore, in this present study, we examined the potential role of cigarette smoking in modulating the surface activation antigen CD28, the numbers of cytotoxic effector T-cells ($CD27^-/CD45RA^+$) and expression of the Tc1-specific chemokine receptor $CXCR_3$ surface antigen on peripheral blood $CD8^+$ lymphocytes. Furthermore, we studied the chemotactic activity of $CD8^+$ T-cells to MCP-1 and compared non-smokers and smokers with and without COPD.

MATERIALS AND METHODS

Subjects

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

None of the subjects were using oral or inhaled corticosteroids, or receiving immunosuppressive treatment or reported any other serious illness or acute viral disease during the two months preceding the test or had tuberculosis or parasite

infections or histories of allergies or asthma. The non-smokers and smokers (≥ 10 pack years and ≤ 40 pack years) with normal chest radiographic and spirometry findings were selected from a population that had undergone an annual health checkup. 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 years) had a history of cough with sputum production and/or dyspnea on most days of the month for at least 3 months a year during > 2 years before the study, and airflow limitation on spirometry (FEV_1/FVC < 70 % and FEV_1 < 80 % of predicted value) as defined by the GOLD initiative [18, 19]. The airflow limitation in these patients was irreversible as shown by a negative immediate response to inhalation of 200 µg of albuterol (≤ 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, Germany.

Pulmonary Function Study

Spirometric parameters and lung volumes were measured according to the recommendations of the American Thoracic Society [20] using a body plethysmograph (Jaeger, Masterlab, Würzburg, Germany) (Table 1) and were expressed as percent of predicted using the prediction formula of Goldman and Becklage [21].

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 WBC count (see Table 2). Peripheral blood mononuclear cells (PBMCs) were separated from acid citrate dextrose (ACD) venous blood and obtained after Ficoll-Paque gradient centrifugation. T cells were isolated from mononuclear cells (MNC) by immunomagnetic depletion of CD14, CD16, CD56 and HLA Class IIDR/DP (B cells, NK cells, monocytes, granulocytes) with a T-cell isolation kit (Dynal Biotech, Hamburg, Germany; cat # 113.11) by negative selection. The selected cells were routinely ≥ 95 % $CD3^+$. To obtain purified $CD8^+$ T-cells, we used the $CD8^+$ isolation kit (Dynal, Hamburg, Germany; cat # 113.05) for rapid immunomagnetic separation of $CD4^+$ T cells and $CD8^+$ lymphocytes according to the manufacturer´s instructions. Purity of the $CD8^+$ T-cells determined by flow cytometry was routinely ≥ 98 %.

$CD8^+$ T-cell chemotaxis assay

The chemotaxis assay was a 96-well microchamber as previously described [22]. The chemoattractant MCP-1 was diluted in RPMI 1640 medium containing 1 % FCS to final concentrations of 0.125 µg/ml and 0.25 µg/ml and 50 µl placed in the lower chamber. Purified $CD8^+$ T-cells were resuspended at a concentration of 5×10^6 cells/ml in RPMI / 1% FCS and incubated for 1 h at 37°C in an atmosphere containing 5 % CO_2 . A cell suspension of 50 µl (2.5×10^5 cells) was added to the upper part of the chamber separated from the lower chamber by a 5-µm-pore-size polycarbonate, polyvinylpyrrolidone-free filter (Applichem, Darmstadt, Germany; cat# K50SH58050) coated with mouse type IV collagen (BD Biosciences, Bedford, England, cat# 354233). 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 USA, cat# 130832). 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 (Nikon diaphot, ELWD 0.3 (OD 75 mm) with a 10 x 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 numbers of migrated cells in the samples.

Four-color fluorescence activated cell sorting (FACS) analysis and gating strategy

Whole blood (100 µl/tube) was incubated with $CXCR_3^+$-conjugated FITC-Ab (R&D Systems, cat# FAB160F), $CD4^+$-conjugated FITC-Ab, $CD45RA^+$-conjugated FITC-Ab, $CD28^+$-conjugated PE-Ab, $CD27^+$-conjugated PE-Ab, $CD3^+$-conjugated ECD-Ab or $CD8^+$-conjugated PC5-Ab (Beckman Coulter, Krefeld, Germany) for 20 minutes in darkness at RT according to the study protocol. FITC-, PE- and PC5-conjugated Isotype-IgG control (cat# IM1672) and Isotype-IgG-ECD control (cat# IM2714) were purchased from Beckman Coulter, Krefeld, Germany. The red blood cells were lysed using lysing solution for 20 minutes (Beckman Coulter, Krefeld, Germany; cat# IM3514). The samples were then vortexed, and centrifuged for 5 minutes at 1700 rpm and 4°C; the supernatant was discarded, the pellet resuspended in 1 ml PBS; 50 000 cells were analysed by flow cytometry in each test. Cells were initially gated on the basis of forward scatter and side scatter characteristics, with gates set to remove debris and platelets. Results were expressed as a percentage of cells exhibiting positive fluorescence.

DATA ANALYSIS

Data are reported as mean ± SEM. A Kruskal-Wallis one-way analysis of variance was used to evaluate significant differences between groups, and, when significance was found, *post hoc* between-group analysis was performed with the Student-Newman-Keuls test. P values < 0.05 were 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 non-smokers ($p < 0.05$). There was a decrease in total lymphocytes in smokers with COPD compared with smokers without COPD and controls which was statistically significant when expressed as 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±2.1 %, $p<0.01$; *$CD8^+$*: 22.7±1.6; $p<0.01$) compared with non-smokers (*$CD4^+$: 60.5*±2.0, *$CD8^+$*: 34.2±2.0) and smokers without COPD (*$CD4^+$:* 65.9±2.5 %, $p<0.05$; *$CD8^+$*: 29.8±2.1; $p<0.05$; graph not shown). As Fig. 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 with non-smokers (1.9±0.2, $p<0.01$).

T_H1 – T_H2 – differentiation of $CD8^+$ T-lymphocytes in peripheral blood

Fig. 2 (Panel A) shows original flow cytometry analyses based on PC-5-conjugated-$CD8^+$ T-lymphocytes and FITC-conjugated-$CXCR_3^+$ T-cell populations from a non-smoker and a smoker without COPD and from a smoker with COPD. Panel B summarises the data of parallel stainings for ECD-conjugated $CD3^+$ T-cells and PC5-conjugated $CD8^+$ and FITC-conjugated $CXCR_3^+$ dot blots and shows flow cytometry analysis results of $CXCR_3^+$ peripheral blood T-lymphocytes expressed as a percentage of total $CD8^+$ T-cells. All events above the isotype control were defined as $CXCR_3^+$.

There was a significant increase in the percentage of $CXCR_3^+$ / $CD8^+$ T-lymphocytes in smokers with COPD (70.1±1.8 %) compared with non-smokers (60.7±2.5 %; $p<0.05$) and smokers without COPD (60.9±2.6; $p<0.05$). In contrast, there was no statistical difference between $CXCR_3^+$ / $CD8^+$ T-lymphocytes in smokers without COPD and non-smokers ($p>0.05$, NS) indicating that the increased number of $CXCR_3^+$ / $CD8^+$ T-cells in COPD is smoking independent (Fig. 2 B). 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 costimulatory 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 non-smoker, a smoking individual, and from a subject with COPD as shown in Fig. 3 A. Summarised individual values of flow cytometry results of $CD28^+$ peripheral blood T-lymphocytes were expressed as percentage of total $CD8^+$ T-cells. As Fig. 3 B shows, there was a

significant increase in the percentage of $CD28^+$ / $CD8^+$ T-cells in smokers without COPD (72.9±3.7; $p<0.05$) and in subjects with COPD (75.4±3.9 %; $p<0.05$) compared with non-smokers (61.0±2.7 %). In contrast, there was no significant difference between $CD28^+$ / $CD8^+$ T-lymphocytes in smokers with and without COPD ($p>0.05$, NS) indicating 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 ($CD8^+$ T-lymphocyte migration of smokers: 1.58±0.23; COPD: 1.89±0.54; non-smokers: 1.79±0.15; NS).

However, as Fig. 4 shows, there was decreased chemotactic activity of $CD8^+$ T-lymphocytes to MCP-1 at a concentration of 0.125 µg/ml in smokers without COPD ($CD8^+$ T-lymphocyte migration: 6.3±0.8) and in smokers with COPD ($CD8^+$ T-lymphocyte migration: 6.7±0.7) compared with non-smokers ($CD8^+$ T-lymphocyte migration: 9.9±1.0; $p<0.05$). There was no statistical difference between smokers with and without COPD (NS) indicating a smoking-dependent decreased chemotactic activity of $CD8^+$ T-cells to MCP-1.

At the higher concentration of 0.25 µg/ml 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 non-smokers (10.7±1.4; $p>0.05$).

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

In this study we 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 $CD8^+$ and FITC-conjugated $CD45RA^+$ and PE-conjugated $CD27^+$ dot blots (Fig. 5 A). In Fig. 5 B $CD27^-/CD45RA^+$ peripheral blood $CD8^+$ T-lymphocytes are expressed as percentage of total $CD8^+$ T-cells from individual patients. The data presented are derived from separate experiments using cells from 13 non-smokers, 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±2.4 %) and from smokers with COPD (23.5±4.3) compared with non-smokers (8.4±1.2 %; $p<0.05$ vs smokers; $p<0.01$ vs COPD) indicating 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 this study we 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 non-smokers (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 non-smokers [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. Our smoking-independent results in COPD may be consistent with a study by Amadori et al., [26] who presented evidence explaining why smokers may develop COPD, and suggest that individuals with a genetically determined increase in the $CD8^+$ population might be more susceptible to further $CD8^+$ 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 our 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, but in this study all COPD patients were non-smokers [29]. Kim and collegues [6] studied subgroups of smokers with COPD and reported that a reduced diffusing capacity of the lung for carbon monoxide (DLCO) per unit of alveolar volume (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 DLCO/VA, suggesting that T-lymphocyte abnormalities might be involved in the pathogenesis of airflow limitation.

Recent evidence suggests that $CD8^+$ T-cells implicated in COPD are of the type 1 phenotype [30]. Indeed, in our study we were able to demonstrate a smoking-independent increase in the Tc1-specific $CXCR_3^+$ surface antigen of peripheral blood $CD8^+$ lymphocytes in smokers with COPD compared to smokers without airflow limitation (Fig. 2). Release of $CXCR_3$ -activating chemokines attracts Tc1 cells into the lungs followed by expression of IFNγ, which may lead to accumulation of activated Tc1 cells in the peripheral lung [30]. The specificity of this effect was confirmed by Saetta and coworkers who found increased numbers of $CXCR_3^+$ T-cells in both epithelium and submucosa of the airways in smokers with COPD compared to

controls [1]. Interestingly, Tzanakis et al. found a decrease in IFNγ-producing $CD8^+$ cells (Tc1) in sputum from smokers with COPD compared with smokers and non-smokers [25]. This is consistent with findings of Leckie and coworkers who found a lower percentage of $CD8^+$ lymphocytes expressing $CXCR_3^+$ in induced sputum from COPD patients compared with non-smokers [10] indicating that sputum results do not always reflect changes of T-cell phenotype seen in the airways and in peripheral blood. Xie and coworkers demonstrated that $CXCR_3$ played a critical role in T cell transmigration to sites of inflammation [9]. These findings provide further evidence that dysregulation of Tc1 agonist chemokines and their cognate receptors such as $CXCR_3$ 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., 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 FEV_1 % of predicted [7], 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. showed lower numbers of $CD8^+$ T-cell activation markers ($CD57^+$ and $CD28^+$) in BAL fluid of smokers compared with non-smokers [32]. In comparison to previous studies in the airways, in this study we demonstrated that in peripheral blood cigarette smoking increased the expression of the surface activation marker CD28 on $CD8^+$ T lymphocytes in smokers with and without airway obstruction compared to non-smokers (Fig. 3). If 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], which 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].

We also investigated the systemic immunotoxicity of cigarette smoking as measured by the cytotoxic effector ($CD27^-/CD45RA^+$) peripheral blood $CD8^+$ T-lymphocytes. We found that cigarette smoking increased the percentage of cytotoxic effector $CD8^+$ T lymphocytes in peripheral blood of smokers and of COPD patients compared with non-smokers (Fig. 5). This subpopulation of $CD8^+$ lymphocytes causes lysis of target cells by two mechanisms: (1) by membranolysis, in which secreted molecules, such as perforin and granzymes, form pores in the membrane of target cells [17] and (2) by 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 µg/ml (Fig. 4) but did not change the spontaneous migration of $CD8^+$ lymphocytes of smokers with and without COPD compared with non-smokers. Interestingly, these smoking induced significant changes in chemotactic activity to MCP-1 could not be found at a higher concentration of 0.25 µg/ml, indicating 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 $CD8^+$ 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, our findings demonstrate that surface antigen modification on peripheral blood $CD8^+$ lymphocytes plays an important role in the pathogenesis of COPD, and that this may be generated only in part by cigarette smoking. The scientific significance and validity of our results may be influenced by the small sample sizes, age, smoking history and gender in each group. These effects could potentially affect the clinical relevance of our findings and should therefore be addressed in future studies.

ACKNOWLEDGMENT

[1]Andrea Koch was supported by grants from the *Deutsche Forschungsgemeinschaft (DFG)*, Bonn, Germany (KO-1788/3-1) and the *Lise-Meitner-Habilitations-Program* of the *Ministerium für Schule, Wissenschaft und Forschung des Landes Nordrhein Westfalen* (44-6037.5), Germany, by Bayer Ltd, Leverkusen, Germany and Bayer Research Centres in England and Kyoto, Bayer Yakuhin Ltd, Kyoto, Japan.

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TABLE 1

CHARACTERISTICS OF HEALTHY NON-SMOKERS, SMOKERS AND SUBJECTS WITH COPD

	Non-Smokers	*Smokers*	*COPD*
n	**13**	**14**	**12**
Age (yr)	**48.3 ± 2.7**	**47.4 ± 4.3**	**56.0 ± 2.1**
Gender (m:f)	**7 : 6**	**8 : 6**	**7 : 5**
FEV_1 (% pred.)	**100.6 ± 3.1**	**104.5 ± 3.6**	**46.7 ± 3.9 ****
FEV_1/FVC (% pred.)	**84.3 ± 1.5**	**84.7 ± 2.2**	**54.3 ± 2.7 ****
FVC (% pred.)	**100.9 ± 2.8**	**106.1 ± 4.5**	**68.8 ± 4.4 ****
RV/TLC (% pred.)	**28.9 ± 1.8**	**33.6 ± 3.4**	**66.4 ± 2.8 ****
RV (% pred.)	**110.0 ± 7.0**	**118.6 ± 11.1**	**211.5 ± 8.3 ****
IC (% pred.)	**86.6 ± 3.2**	**89.4 ± 7.4**	**60.7 ± 6.0 ****
Pack years	**-**	**33.8 ± 0.5**	**35.3 ± 2.8**

** $p<0.01$ vs smokers and non-smokers by Student-Neumann-Keuls test

<u>ABBREVIATIONS IN TABLE 1:</u>

FEV_1 Forced Expiratory Volume in 1 Sec

FVC Forced Vital Capacity

RV Residual Volume

TLC Total Lung Capacity

IC Inspiratory Capacity

TABLE 2

PERIPHERAL BLOOD CELL COUNTS

	Non-Smokers	*Smokers*	*COPD*
n	**13**	**14**	**12**
Leukos (x 10^3 /µl)	**6.5 ± 0.3**	**7.8 ± 0.8**	**8.8 ± 0.7**
Lymphos (x 10^3 /µl)	**2.0 ± 0.1**	**2.3 ± 0.3**	**1.9 ± 0.1**
Lymphos (% Leukos)	**31.3 ± 2.3**	**29.7 ± 1.5**	**22.9 ± 1.5 ****
Eos (x 10^3 /µl)	**0.16 ± 0.03**	**0.15 ± 0.02**	**0.24 ± 0.05**
Eos (% Leukos)	**2.47 ± 0.4**	**2.01 ± 0.2**	**2.85 ± 0.4**
Neutros (x 10^3 /µl)	**3.79 ± 0.3**	**4.67 ± 0.5**	**5.79 ± 0.5 §**
Neutros (% Leukos)	**57.5 ± 26**	**59.9 ± 1.9**	**65.2 ± 1.7 §**
Monos (x 10^3 /µl)	**0.4 ± 0.03**	**0.4 ± 0.06**	**0.5 ± 0.06**
Monos (% Leukos)	**5.63 ± 0.36**	**5.16 ± 0.50**	**6.12 ± 0.46**
Baso (x 10^3 /µl)	**0.05 ± 0.007**	**0.06 ± 0.009**	**0.07 ± 0.015**
Baso (% Leukos)	**0.90 ± 0.12**	**0.92 ± 0.13**	**0.92 ± 0.13**

** p<0.01vs smokers and non-smokers by Student-Newman-Keuls test
* p<0.05 vs smokers and non-smokers by Student-Newman-Keuls test
§ p<0.05 vs non-smokers by Student-Newman-Keuls test

LEGEND TO FIGURES:

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 non-smokers, 14 smokers without and 12 smokers with COPD. There was a significant increase in $CD4^+$ / $CD8^+$ ratio in smokers with COPD compared with smokers without COPD

and non-smokers while no difference was found between smokers without COPD and non-smokers.

Figure 2

Panel A: Representative original FACS analyses of ECD-conjugated $CD3^+$ T-cells and PC-5-conjugated-$CD8^+$ and FITC-conjugated-$CXCR3^+$ populations of a non-smoking and a smoking individual and of a subject with COPD.

Panel B: summarises individual results of FACS analysis of ECD-conjugated $CD3^+$ T-cells and PC5-conjugated $CD8^+$ and FITC-conjugated $CXCR_3^+$ dot blots of 13 non-smokers, 14 smokers without and 12 smokers with COPD. There was a significant increase in the percentage of $CXCR_3^+$ T-lymphocytes in relation to total $CD8^+$ T-cells in smokers with COPD compared with non-smokers and smokers without COPD. In contrast, there was no significant difference between smokers without COPD and non-smokers.

Figure 3

Panel A: Representative original FACS analyses based on ECD-conjugated $CD3^+$ / PC-5-conjugated-$CD8^+$ and PE-conjugated-$CD28^+$ populations of a non-smoker and a smoker with and without COPD.

Panel B: $CD28^+$ peripheral blood T-lymphocytes expressed as a percentage of $CD8^+$ T-cells of 13 non-smokers, 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 non-smokers while no differerence was found between smokers with and without COPD.

Figure 4

$CD8^+$ T-cell chemotaxis assay of MCP-1 (0.125 µg/ml) stimulated peripheral blood T-lymphocytes expressed as $CD8^+$ T-lymphocyte migration/field using cells from 13 non-smokers, 14 smokers without and 12 smokers with COPD. There was a significant decreased chemotactic activity of $CD8^+$ T-lymphocytes to MCP-1 from smokers with and without COPD compared with non-smokers but no statistical difference between smokers with and without COPD..

Figure 5

Panel A: Representative original FACS analyses of PC5-conjugated $CD8^+$ and PE-conjugated $CD27^+$ T-cells and FITC-conjugated-$CD45RA^+$ T cell population from a non-smoker, a smoker with and a smoker without COPD.

Panel B: Panel B summariees the individual values of parallel colour stainings of $CD27^-$ and $CD45RA^+$ peripheral blood T-lymphocytes expressed as percentage of $CD8^+$ T-cells from 13 non-smokers, 14 smokers without and 12 smokers with COPD. There was a significant increase in the percentage of $CD27^-/CD45RA^+$ $CD8^+$ T-lymphocytes in relation to total $CD8^+$ T-cells of smokers with and without COPD compared with non-smokers. In contrast there was no statistical difference between $CD27^-/CD45RA^+$ T-lymphocytes in relation to $CD8^+$ T-cells in smokers with and without COPD.

Fig. 1

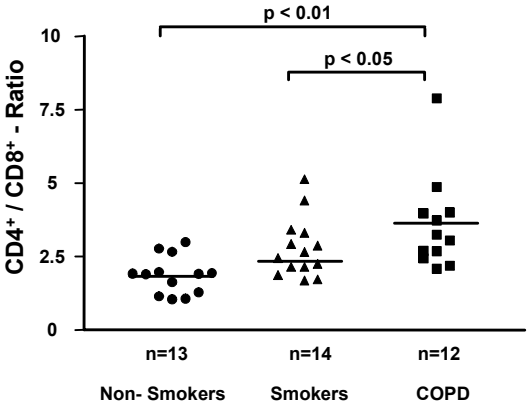
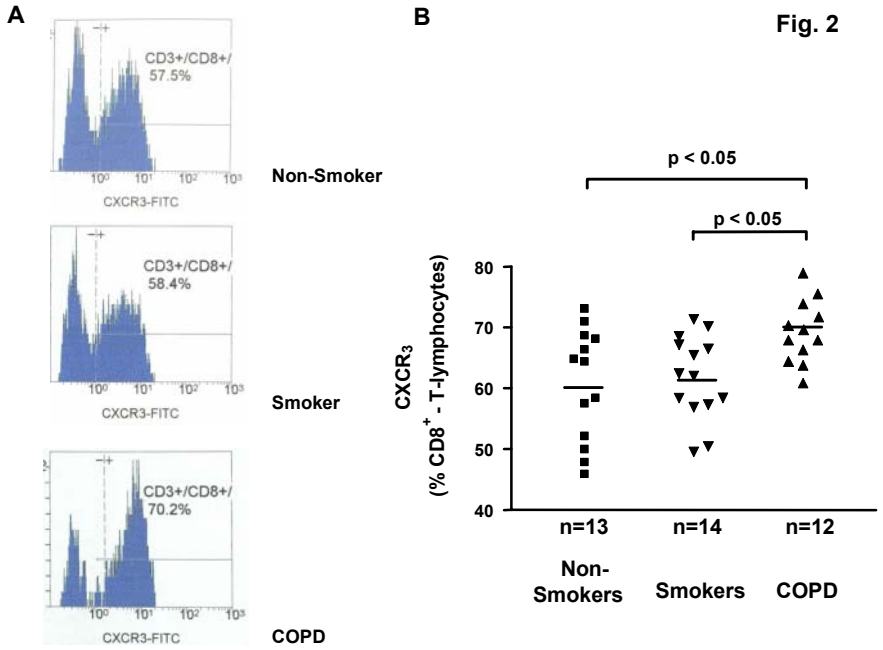

p < 0.01
p < 0.05
10
7.5
5
2.5
0
CD4+ / CD8+ - Ratio
n=13
n=14
n=12
Non- Smokers
Smokers
COPD

Fig. 2


A
CD3+/CD8+/
57.5%
CXCR3-FITC
Non-Smoker
CD3+/CD8+/
58.4%
CXCR3-FITC
Smoker
CD3+/CD8+/
70.2%
CXCR3-FITC
COPD
B
p < 0.05
p < 0.05
CXCR3
(% CD8+ - T-lymphocytes)
80
70
60
50
40
n=13
n=14
n=12
Non-
Smokers
Smokers
COPD

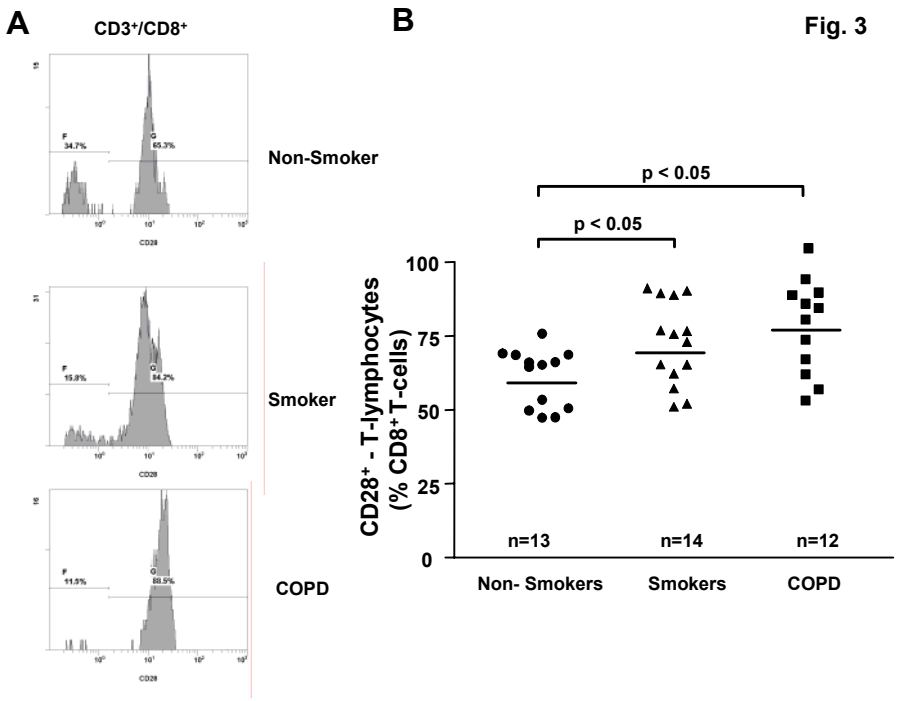
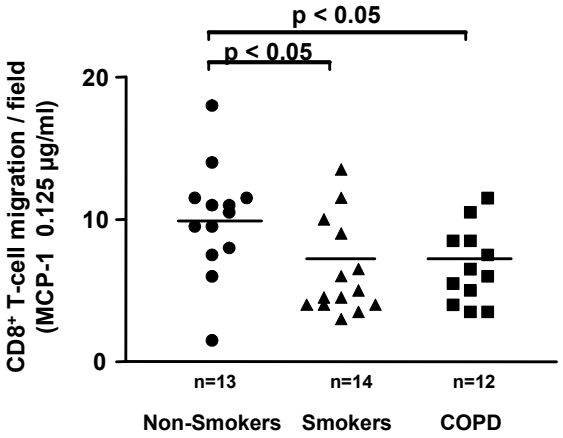

A
CD3+/CD8+
Non-Smoker
Smoker
COPD
B
p < 0.05
p < 0.05
CD28+ - T-lymphocytes
(% CD8+ T-cells)
100
75
50
25
0
n=13
n=14
n=12
Non- Smokers
Smokers
COPD

Fig. 3

Fig. 4


p < 0.05
p < 0.05
CD8+ T-cell migration / field
(MCP-1 0.125 µg/ml)
20
10
0
n=13
n=14
n=12
Non-Smokers
Smokers
COPD

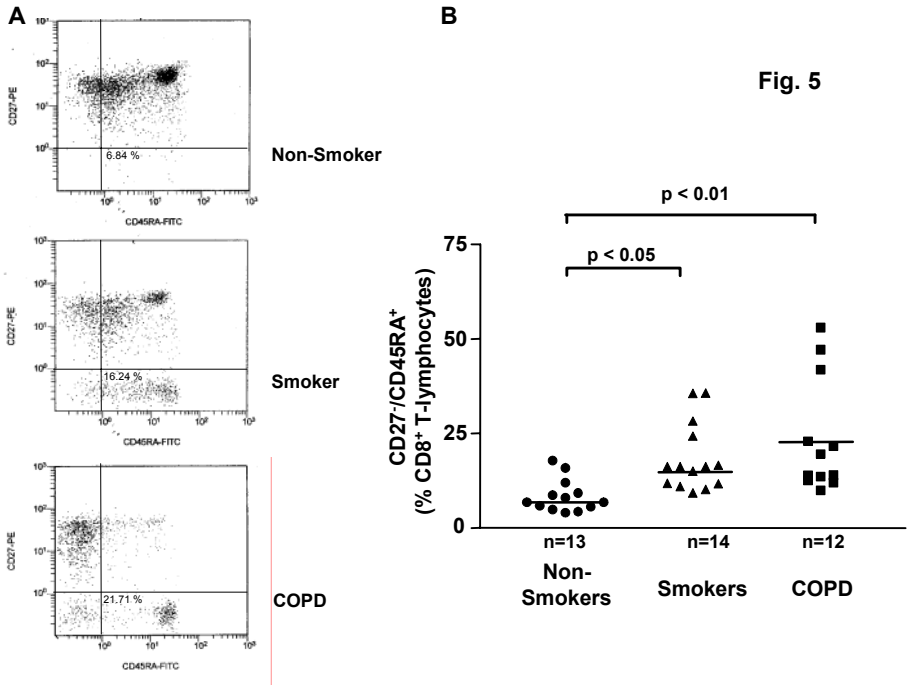

A
B
CD27-PE
CD45RA-FITC
6.84 %
Non-Smoker
16.24 %
Smoker
21.71 %
COPD
p < 0.01
p < 0.05
75
50
25
0
CD27-/CD45RA+
(% CD8+ T-lymphocytes)
n=13
n=14
n=12
Non-
Smokers
Smokers
COPD

Fig. 5