Increased levels of interleukin-16 in the airways of tobacco smokers: relationship with peripheral blood T lymphocytes

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Abstract

***Background*—The mechanisms behind the development of systemic immunomodulation among tobacco smokers are not fully understood, but several studies have indicated a role for CD8+ and/or CD4+ T cells. Interleukin (IL)-16, a cytokine released from inflammatory cells as well as bronchial epithelial cells, can recruit and activate CD4+ T cells. A study was undertaken to establish whether the IL-16 level is increased in the airways of tobacco smokers and to determine whether airway levels of IL-16 are related to the number and function of systemic T lymphocytes.**

***Methods*—Bronchoalveolar lavage (BAL) fluid was collected from eight never smokers and 18 tobacco smokers without clinical airway symptoms, and from 16 tobacco smokers with clinical airway symptoms. Interleukin-16 protein levels in BAL fluid were determined using enzyme-linked immunosorbent assay (ELISA). Peripheral blood was collected for determination of CD4+ T cell content using flow cytometry. The responsiveness of systemic lymphocytes in smokers was assessed by measuring the proliferative response of peripheral blood lymphocytes to the superantigen staphylococcus enterotoxin A (SEA).**

***Results*—The IL-16 protein level in the BAL fluid was significantly higher in tobacco smokers than in non-smokers. However, among tobacco smokers the IL-16 level was similar in asymptomatic smokers and in those with airway symptoms. The level of IL-16 in the BAL fluid of smokers correlated negatively with the percentage of CD4+ T cells and positively with superantigen stimulated lymphocyte proliferation in peripheral blood.**

***Conclusions*—In tobacco smokers the airway IL-16 level is increased and it is possible that this increase in IL-16 influences systemic immunomodulation by altering the number and responsiveness of systemic T lymphocytes.**

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Keywords: interleukin 16; tobacco; smoking

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Tobacco smoking is characterised by profound immunological changes which impair host defence and increase susceptibility to infection.[1] Tobacco smoking is also a significant risk factor for diseases in the respiratory tract of infectious, malignant and inflammatory aetiology, and also for diseases in organs other than the lung.[2] Smoking related systemic immunomodulation has previously been investigated in terms of humoral and cell mediated immunity[1] and it is known that smoking alters the course of diseases such as AIDS[3] and influenza,[4] as well as the incidence of allergic conditions.[5] All these diseases are characterised by changes in subsets of T lymphocytes and tobacco smoking has therefore been studied in the context of its effects on T lymphocytes and cytokines related to these cells.[1 6–10]

Local and systemic T lymphocytes may also be involved in the pathogenesis of chronic bronchitis, a frequently diagnosed consequence of tobacco smoking which is characterised by increased numbers of CD8+ T cells as well as activated (CD25+) T cells in the airway epithelium.[6 7] Tobacco smoking may also increase CD8+ T cells in peripheral airways[8] and it is known to alter the number and function of systemic lymphocytes. Under certain conditions tobacco smoking increases the percentage of peripheral blood CD4+ T cells[9 10] but also impairs the responsiveness of peripheral blood lymphocytes.[11–13] However, little is known about the mediators connecting tobacco smoking to the status of these systemic T lymphocytes.

Interleukin (IL)-16, previously known as lymphocyte chemoattractant factor, is a recently characterised cytokine produced in the airways mainly by CD8+ T cells and bronchial epithelial cells.[14 15] Being a ligand for CD4, IL-16 causes effects exclusively restricted to cells bearing the CD4 receptor including CD4+ T cells and subsets of macrophages and eosinophils.[15 16] In vitro, IL-16 is a selective chemoattractant for CD4+ T cells.[17] In addition, it has been shown to induce expression of the IL-2 receptor on CD4+ T cells, thereby increasing the activity and proliferation of these cells.[18] This effect, however, seems to be bimodal and depends on the type of co-stimulus.[19 20]

Several studies have suggested an important role for IL-16 in airway inflammation. In sensitised mice airways in vivo, allergen exposure increases epithelial release of IL-16 protein.[21] Treatment with an IL-16 antibody inhibits the development of airway smooth muscle hyperresponsiveness as well as the upregulation of allergen specific immunoglobulin (Ig)E.[21] In the airways of atopic subjects with asthma,

allergen challenge also increases the IL-16 protein level as indicated in bronchoalveolar lavage (BAL) fluid; this may be due to an increased epithelial release of IL-16 protein.[22 23] IL-16 accounts for approximately 80% of the chemotactic activity for lymphocytes in the BAL fluid of allergen challenged atopic subjects.[22] Thus, there is some evidence that inflammatory stimuli may release IL-16 in the airways which subsequently modulates the status of lymphocytes locally and/or systemically. However, at present, there are no data available on IL-16 levels in the airways of tobacco smokers, whether or not they have airway symptoms.

The primary aim of this study was to evaluate whether the IL-16 protein level is increased in the airways of non-allergic tobacco smokers and whether the IL-16 level is higher in smokers with clinical symptoms than in those without symptoms. Since both tobacco smoking and IL-16 can modulate the number and the function of T lymphocytes, the secondary aim was to determine how the airway IL-16 level in tobacco smokers relates to systemic immunomodulation by characterising its relation to the number and responsiveness of peripheral blood T lymphocytes.

Table 1 Clinical characteristics of eight never smokers and 34 tobacco smokers

	Smoking status	*CB*	*Pack years*	*Cigs/day*	*Age*	*Sex*	*BAL fluid IL-16 (pg/ml)*
	−	−	−	−	42	M	509
	−	−	−	−	42	M	778
	−	−	−	−	57	M	1258
	−	−	−	−	60	F	964
	−	−	−	−	64	F	772
	−	−	−	−	46	M	1094
	−	−	−	−	36	M	753
	−	−	−	−	60	F	588
Mean (SD)					51 (11)	5M/3F	840 (250)
	+	−	57	30	58	M	1668
	+	−	15	10	45	F	1351
	+	−	45	30	45	M	342
	+	−	19.5	15	40	F	1485
	+	−	37	20	51	M	2323
	+	−	35	20	48	F	2975
	+	−	18	15	37	F	1314
	+	−	18	15	42	F	1148
	+	−	27	20	44	F	862
	+	−	38	20	52	M	519
	+	−	21.5	17	40	F	2200
	+	−	31.5	25	41	F	1223
	+	−	34	20	48	M	5066
	+	−	17	10	51	F	675
	+	−	34	20	48	M	807
	+	−	15	15	35	F	994
	+	−	21	15	39	M	2834
	+	−	29.5	15	56	F	269
	+	+	35	20	62	F	807
	+	+	24.5	15	49	F	5008
	+	+	39	20	63	F	3056
	+	+	15.5	10	53	F	3927
	+	+	30	20	44	F	1961
	+	+	21	15	51	F	1591
	+	+	36	20	51	M	834
	+	+	21	20	58	F	1631
	+	+	28.5	15	57	M	639
	+	+	22.5	15	56	F	435
	+	+	26	20	38	F	783
	+	+	39	20	53	F	2098
	+	+	32	20	49	M	266
	+	+	25	20	44	F	197
	+	+	30	30	55	F	1546
	+	+	44	20	55	M	3425
Mean (SD)			29 (10)	19 (5)	49 (7)	11M/23F	1655 (1285)

CB = chronic bronchitis; BAL = bronchoalveolar lavage; pack years = cigarettes smoked per day × years of smoking/20.

Methods

STUDY DESIGN

This was a cross sectional study. The subjects were recruited from patient files at the Department of Respiratory Medicine, Sahlgrenska University Hospital, Göteborg and through an advertisement in a daily newspaper. The medical examination and lung function tests took place at the first visit of the subjects. Approximately two weeks later a fibreoptic bronchoscopic examination with BAL was performed. The patients were examined between May 1993 and June 1995. The study was approved by the ethics committee at the University of Göteborg (DNo. 41/96). The subjects gave their consent after both written and oral information, in accordance with the Helsinki declaration of ethics for studies on human subjects.

SUBJECTS

Eight never smokers without airway symptoms were included as control subjects. There were 34 tobacco smokers, all of whom had been smokers for more than 10 years, consuming at least 10 cigarettes per day. We recently presented characteristics other than IL-16 for the group of tobacco smokers.[24] Among the tobacco smokers, 18 subjects were clinically asymptomatic smokers who did not have chronic bronchitis and 16 were smokers with symptoms of chronic bronchitis as defined by the American Thoracic Society.[25] All subjects with chronic bronchitis had a history of two or more infectious exacerbations during the past 12 months.[26]

All asymptomatic subjects, both non-smokers and smokers, had normal lung function defined as forced expiratory volume in one second (FEV_1) >80% predicted. Co-existing chronic airway obstruction, defined as FEV_1 <80% predicted, was allowed only among subjects with chronic bronchitis.

No subject was allowed to use N-acetylcysteine or antihistamines or to undergo vaccination or other immunomodulating treatment during the four weeks before the first examination. No glucocorticoid treatment (oestrogen included)—whether oral, for inhalation, dermal or nasal—or other immunosuppressant treatment was allowed during the three months before the first investigation. All subjects had been free of symptoms of infectious respiratory disease during the four weeks prior to the investigation. The subjects were aged 35–65 years. Individual clinical data are presented in table 1.

Criteria for exclusion were baseline FEV_1 <45% predicted (subjects with chronic bronchitis only), a post-bronchodilator increase in FEV_1 of >15% predicted; abnormal chest radiograph; bronchial hypersecretion caused by other known active pulmonary diseases such as sarcoidosis or cystic fibrosis; known immunodeficiency; α_1-antitrypsin deficiency; or a history of asthma. Patients with known atopy were also excluded, as were patients with concurrent severe diseases of any kind as judged by the clinical investigators.

Ventilatory lung function (FEV_1 % predicted) was measured with a Vitalograph Alpha

(Vitalograph Ltd, Buckingham, UK) in a standardised manner.[27]

FIBREOPTIC BRONCHOSCOPY

Premedication was given as oral diazepam (5–10 mg) together with 0.5–1 ml morphine-scopolamine (10 mg/ml + 0.4 mg/ml) i.m. 45 minutes before bronchoscopy. Local anaesthesia of the oropharynx was achieved with 1% tetracaine (5 ml) sprayed by an aerosol device (DeVilbiss Ltd, Heston, UK), followed by an additional 4–5 ml instillation of tetracaine into the larynx. All bronchoscopies were performed transorally with the subject in the supine position.

BRONCHOALVEOLAR LAVAGE

With the bronchoscope wedged in a middle lobe bronchus, BAL was performed by instilling 2×100 ml sterile phosphate buffered saline (PBS) at 37°C and aspirating the fluid immediately after each instillation. The aspirated BAL fluid was pooled in a siliconised glass container and immediately transported on ice for analysis.

Before analysis the BAL fluid was filtered through a nylon web with a pore size of 100 µm for retention of mucus and cell debris and centrifuged at 250*g* for 10 minutes at +4°C. The BAL supernatant was separated, centrifuged at 10 000*g* for 10 minutes, and stored in aliquots at –70°C. To determine recovery of the epithelial lining fluid volume the BAL albumin levels were measured using a commercial double antibody radioimmunoassay (RIA) (Pharmacia-Upjohn, Uppsala, Sweden).

IL-16 PROTEIN MEASUREMENT IN BRONCHOALVEOLAR LAVAGE FLUID

BAL fluid supernatants from all subgroups were thawed and concentrated 10-fold with Centricon-10 centrifugation filters (10 kD cut off; Amicon Co, Bevery, Massachusetts, USA). The IL-16 protein levels were then measured in a blinded fashion using a commercial IL-16 enzyme-linked immunosorbent assay (ELISA) kit (BioSource International Inc, Camarillo, Colorado, USA).

FLOW CYTOMETRY OF PERIPHERAL BLOOD LYMPHOCYTES

Whole blood from smokers (with ethylenediamine tetra-acetic acid (EDTA) as anticoagulant and in a volume corresponding to about 5×10^5 cells/sample) was incubated at 4°C for 15 minutes with antibodies in the concentrations recommended by the manufacturer. The samples were stained using triple staining with combinations of monoclonal murine antibodies for CD3, CD4, CD8, CD19, and CD56 surface antigens, directly conjugated with the fluorochromes fluorescein isothiocyanate, phycoerthrin, or R-phycoerytrin-Cy5 (all from Dakopatts a/s, Glostrup, Denmark).

Cell analysis was performed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, California, USA) calibrated with Calibrite® beads (Becton-Dickinson). A lymphocyte gate was set manually according to the location in the forward scatter versus side scatter diagram. Negative isotype controls (Dakopatts a/s) were used to set quadrant markers which delineated positive fluorescent staining from non-antigen specific staining. Dot plots and quadrant statistics from three-colour analysis were generated by Lysis II software (Becton-Dickinson). The absolute number of blood lymphocytes was determined using a haematological cell counter (Sysmex-K1000; TOA Medical Electronics Co). All percentages of lymphocyte subpopulations from each individual were divided by the sum of the percentages of CD3+, CD19+ and CD3-56+. This sum includes T cells, B cells, and NK cells and represents all major lymphocyte populations. These adjusted values were then used in all calculations which were expressed as percentage of lymphocytes and as numbers of lymphocytes $\times 10^9$/l.

PERIPHERAL BLOOD LYMPHOCYTE PROLIFERATION

Mononuclear cells were obtained from the peripheral blood of smokers to assess the proliferative responsiveness to a T cell specific superantigen (below). Blood was collected in heparinised tubes and mononuclear cells were prepared by centrifugation on Ficoll-Hypaque (Lymphoprep; Nycomed Pharma a/s, Oslo, Norway) at 827*g* for 10 minutes at room temperature. A total of 1×10^5 cells in a volume of 200 µl was put in the wells of a microtitre plate with round bottom wells (Nuclon; Nunc a/s, Roskilde, Denmark). Cells were incubated in Iscoves' medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 10% human AB serum (Sera-Lab, Sussex, UK), and gentamicin (100 U/ml; Schering-Plough Int, Kenilworth, New Jersey, USA) in 5% CO_2 at 37°C. The superantigen staphylococcus enterotoxin A (SEA; final concentration 5 ng/ml, obtained from Toxin Technology, Inc, Sarasota, Florida, USA) was used for stimulation of the cells in triplicate of wells. Cells without any stimulus (in triplicates) were used as negative controls. Under these conditions the cells were incubated for 48 hours, 150 µl of the culture supernatant was replaced by fresh media (with or without stimulus), and the plate was incubated for another 24 hours. ^{3}H-thymidine (Amersham International plc, Amersham, UK) was then added and the plate was incubated over night. The cells were harvested (96-well plate cell harvester, INOTECH, Dotticon, Switzerland) and radioactivity measured as counts per minute (cpm) using a β-counter (Matrix 96; Packard Instrument Co Inc, Chicago, Illinois, USA). The results were presented as stimulatory index (SI) = cpm of stimulated cells/cpm of unstimulated cells. Because SEA, like any other superantigen, activates cells bearing T cell receptor (TCR) only, the changes in proliferation of mononuclear cells are expressed as proliferative response of lymphocytes throughout the paper.

ANALYSIS OF DATA

Unless otherwise stated, data are presented as mean (SE). The StatView 4.01 (Abacus Concepts, Berkeley, California, USA) software was used. For comparisons between groups the

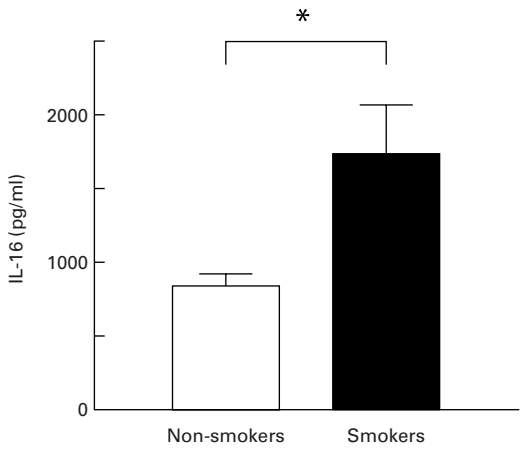

*Figure 1 Concentration of IL-16 protein in concentrated (×10) BAL fluid from 34 tobacco smokers and eight never smokers using ELISA. *p = 0.04 (Student's unpaired one way t test). Data are mean (SE).*

Student's unpaired *t* test was performed. The Spearman rank correlation coefficient was calculated to investigate correlations between clinical variables.

Results

BAL IL-16 PROTEIN LEVEL AND TOBACCO SMOKING

The mean (SD) FEV_1 (% predicted) was 107 (8) in never smokers and 92 (3) in smokers. The mean (SD) albumin level in the BAL fluid was 59.6 (19.4) mg/l in eight never smokers and 53.1 (18.9) mg/l in 34 smokers (two way *t* test: p = 0.4).

The IL-16 protein level was detectable in the concentrated BAL fluid of all subjects included in the study (table 1). In tobacco smokers the IL-16 protein level in BAL fluid was significantly higher than in BAL fluid from non-smokers (fig 1). There was no evident correlation between the level of IL-16 in the BAL fluid of smokers and the past (IL-16 vs pack years (cigarettes smoked per day × years of smoking/20)) or current (IL-16 vs cigarettes/day) number of cigarettes smoked (data not shown). There was no statistically significant difference in IL-16 levels in the BAL fluid from asymptomatic smokers (1560 (280) pg/ml) and smokers with chronic bronchitis (1760 (360) pg/ml; one way *t* test: p = 0.3, n = 16–18).

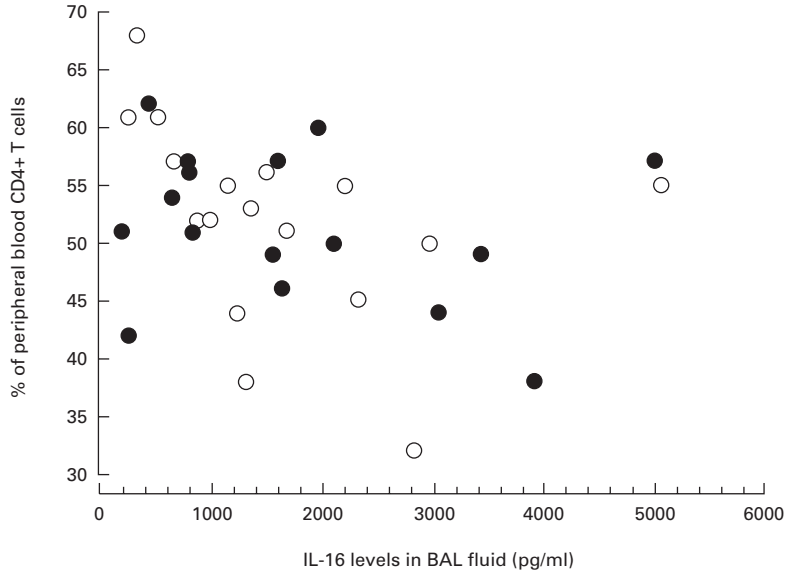

Figure 2 Concentration of IL-16 protein in concentrated (×10) BAL fluid versus percentage of CD4+ T cells in peripheral blood detected by flow cytometry and calculated as a percentage of T lymphocytes in 34 tobacco smokers. The level of IL-16 in the BAL fluid correlated negatively with the percentage of CD4+ T cells in the blood (p = 0.02, r = –0.4). Asymptomatic smokers are shown as open circles and smokers with chronic bronchitis as closed circles.

BAL IL-16 PROTEIN LEVEL VS PERIPHERAL BLOOD CD4+ T CELLS IN TOBACCO SMOKERS

The percentage of CD4+ T cells in peripheral blood from smokers was 52 (1) (absolute number 1.1 (0.06) × 10^9/l). The percentage of CD4+ T cells in peripheral blood correlated significantly and negatively with the IL-16 protein level in the BAL fluid (fig 2). There was no significant difference in percentage of peripheral blood CD4+ T cells between asymptomatic smokers (52(2)%; absolute number: 1.1 (0.1) × 10^9/l) and smokers with chronic bronchitis (51 (2)%; absolute number 1.1 (0.1) × 10^9/l; two-way *t* test: p = 0.8, n = 16–18).

BAL IL-16 PROTEIN LEVEL VS PERIPHERAL BLOOD CD8+ T CELLS IN TOBACCO SMOKERS

The percentage of CD8+ T cells in the peripheral blood of smokers was 26 (1)% (absolute number 0.6 (0.04) ×10^9/l) which did not correlate significantly with the level of IL-16 protein in the BAL fluid (p = 0.3, *r* = 0.1, n = 34). There was no statistically significant difference in the percentage of CD8+ T cells found in the peripheral blood of asymptomatic smokers (25 (1)%, absolute number 0.5 (0.05) × 10^9/l) and in smokers with chronic bronchitis (27 (2)%, absolute number 0.6 (0.06) × 10^9/l) (two way *t* test: p = 0.4, n = 16–18).

BAL IL-16 PROTEIN LEVEL VS PERIPHERAL BLOOD LYMPHOCYTE PROLIFERATION IN TOBACCO SMOKERS

There was a significant and positive correlation between the level of IL-16 protein in the BAL fluid and peripheral blood lymphocyte proliferation induced by SEA (fig 3). The SEA induced peripheral blood lymphocyte proliferation in asymptomatic smokers (SI 246 (57)) was not significantly different from that in smokers with chronic bronchitis (SI 193 (52)) (two way *t* test: p = 0.5, n = 16–18).

Discussion

This study shows that the level of IL-16 protein is higher in the airways of tobacco smokers than in the airways of subjects who have never smoked. However, the IL-16 level was not significantly higher in tobacco smokers with symptoms than in those without. In tobacco smokers the level of airway IL-16 correlated negatively with the percentage of CD4+ T cells in peripheral blood and positively with superantigen induced peripheral blood T lymphocyte proliferation.

Although long term tobacco smoking is known to be a major risk factor for development of chronic bronchitis, bronchial biopsy specimens have shown similar inflammatory cell counts in the airway epithelium of clinically healthy smokers and in healthy never smokers.[28] Both of these groups have been shown to differ qualitatively from smokers with

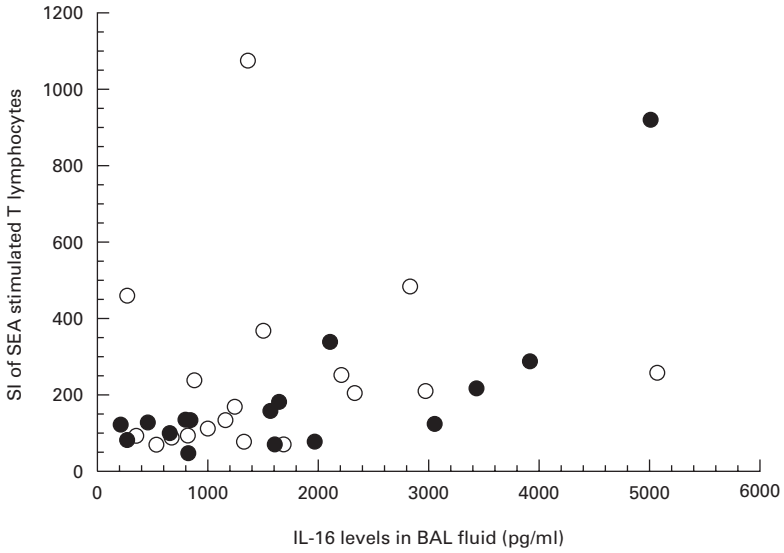

Figure 3 Concentration of IL-16 protein in concentrated (×10) BAL fluid versus proliferative response of peripheral blood T lymphocytes to staphylococcus enterotoxin A (SEA) detected in vitro by ^{3}H-thymidine incorporation in 34 tobacco smokers. The data are presented as stimulatory index (SI), calculated by dividing the proliferation of stimulated cells by the proliferation of unstimulated cells. The level of IL-16 in the BAL fluid correlated positively with the peripheral blood lymphocyte proliferation ($p = 0.01$, $r = 0.5$). Asymptomatic smokers are shown as open circles and smokers with chronic bronchitis as closed circles.

chronic bronchitis.[28 29] The present study identifies one lymphocyte associated cytokine, IL-16, that is increased in the airways of smokers compared with never smokers. The level of IL-16 in the airway was not significantly higher in smokers with chronic bronchitis than in those without, indicating that IL-16 is not directly related to the presence of this disease. This suggests a role for smoking per se rather than subsequent inflammatory events in causing the increased IL-16 protein level in the airways.

We were unable to confirm a correlation between IL-16 airway levels and exposure to tobacco smoke in present (cigarettes/day) or past (pack years) smokers. However, in our study smokers were defined as having a smoking history of at least 10 cigarettes/day and 15 pack years. A wider dose-effect interval will probably be required to detect a (hypothetical) concentration-dependent relationship between exposure to tobacco smoke and IL-16 levels.

This study also shows that the IL-16 level in the BAL fluid of smokers correlates moderately and negatively with the percentage of peripheral blood CD4+ T cells. We did not evaluate the effect of IL-16 on the lymphocyte number in the airway wall, but a previous study has shown that, in asthmatic patients, epithelial expression of IL-16 protein and mRNA correlates with CD4+ cell infiltration in the airway wall.[23] The data in our study are thus compatible with the hypothesis that IL-16 released in the airways modulates the number of systemic CD4+ T cells. IL-16 may act through its selective chemotactic effect to cause recruitment of CD4+ T cells into the airway wall, thereby depleting CD4+ T cells from the peripheral blood. The IL-16 level in the airways was not correlated with the percentage of CD8+ T cells in the peripheral blood, which supports the hypothesis that IL-16 has a selective effect on systemic CD4+ T cells only.

We have also shown that the IL-16 level in the BAL fluid of smokers correlates moderately and positively with the proliferative response to the superantigen SEA of peripheral blood lymphocytes in vitro. Previous in vitro data on effects of IL-16 on lymphocyte proliferation have indicated that, depending on the type of co-stimulus present, IL-16 causes either an increase in CD4+ T cell proliferation or a transient anergy in CD4+ T cells.[19 20] In smokers the increased airway level of IL-16 may thus potentiate the proliferative response of systemic T lymphocytes to bacterial components such as SEA. This indicates that the weakened responsiveness of T cells, a characteristic of tobacco smoking,[11 30] may in fact be counteracted by IL-16.

Population based data on peripheral blood indicate that chronic tobacco smoking increases systemic CD4+ T cells.[9 10] By contrast, the function of CD4+ T cells is impaired in tobacco smokers. This is indicated by the decreased responsiveness of peripheral blood lymphocytes in smokers[11] as well as by the capacity of nicotine or compounds of cigarette tar to induce T cell anergy in vivo[30] and in vitro.[31] A suppressive effect of tobacco smoking on T cell functions is also indicated indirectly by a decreased level of immunoglobulins, except for IgE, in blood from smokers,[12 13] as well as by a lower incidence of allergy among smokers.[13] The results of our study suggest that, in smokers, the airway level of IL-16 protein may determine the decreased percentage of systemic CD4+ T cells and the increased responsiveness of systemic T lymphocytes. These effects of IL-16 are in fact opposite to the previously reported effects of tobacco smoking. It is not yet known whether the increased airway level of IL-16 in tobacco smokers constitutes a defence mechanism against smoking related systemic immunomodulation.

In conclusion, IL-16, a cytokine capable of recruiting and activating CD4+ T cells, is increased in the airways of smokers. The correlation of airway levels of IL-16 with the percentage of CD4+ T cells and the responsiveness of peripheral blood lymphocytes suggest that the increased IL-16 level may alter systemic immunomodulation by influencing the status of systemic T lymphocytes.

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