“Ars longa,

vita brevis,

occasio praeceps,

experimentum periculosum,

iudicium difficile”

"Konsten är lång,

livet är kort,

tillfället flyktigt,

erfarenheten bedräglig,

omdömet svårt"

Hippokrates
cirka 460-370 f.Kr.

ABBREVIATIONS

7-AAD	7-aminoactinomycin D
Abs	antibodies
AM	aminophylline
AS	asymptomatic smokers
BAL	bronchoalveolar lavage
BALF	BAL fluid
cAMP	cyclic adenosine monophosphate
CB	chronic bronchitis
CD	cluster of differentiation
cDNA	complementary DNA
CI	calcium ionophore
CSE	cigarette smoke extract; i.e. water-soluble tobacco smoke components
COPD	chronic obstructive pulmonary disease
Da	Dalton
DL_{CO}	diffusion capacity for carbon monoxide
DNPH	2,4-dinitrophenylhydrazine
ELISA	enzyme-linked immunosorbent assay
FEV_1	forced expiratory volume in 1 second
FVC	forced vital capacity
GL	glutathione
GOLD	global initiative for COPD
HPRT	hypoxanthine-guanine phosphoribosyltransferase
Iκβ-α	inhibitor of NF-κβ
IL-1β	interleukin-1β
IL-2	interleukin-2
IL-16	interleukin-16
IR	immunoreactivity
LCF	lymphocyte chemoattractant factor
MW	molecular weight
mRNA	messenger RNA

neg negative
NFAT nuclear factor of activated T cells
NF-κβ nuclear factor-κβ
NK cells natural killer cells
NS never-smokers
OFR oxygen free radical

OFRs oxygen free radical scavenger
PDE phosphodiesterase inhibor
% pred. % of predicted
PMA phorbol 12-myristate 13-acetate
pos positive
rh recombinant human

rMFI relative mean fluorescence index
ROS reactive oxygen species
RT-PCR reverse transcriptase-polymerase chain reaction
SD standard deviation
STAT6 signal transducer and activator of transcription 6
TNF-α tumor necrosis factor-α
veh vehikel

This thesis is based on the following papers:

I. A. Andersson, I. Qvarfordt, M. Laan, M. Sjöstrand, C. Malmhäll, G. C. Riise, L.-O. Cardell and A. Lindén
Impact of tobacco smoke on interleukin-16 protein in human airways, lymphoid tissue and T lymphocytes
Clinical and Experimental Immunology, 2004 Oct;138(1):75-82.

II. A. Andersson, A. Bossios, C. Malmhäll, M. Sjöstrand, M. Eldh, B-M. Eldh, P. Glader, B. Andersson, I. Qvarfordt, G.C. Riise and A. Lindén
Effects of tobacco smoke on IL-16 in $CD8^+$ cells from human airways and blood: a key role for oxygen free radicals?
American Journal of Physiology, Lung Cellular and Molecular Physiology 2011 Jan;300(1):L43-55.

III. A. Andersson [*)], A. Bossios [*)], C. Malmhäll, B. Houltz, M. Sjöstrand, I. Qvarfordt and A. Lindén
Decrease in Interleukin-16-expressing NK cells in the Blood of Long-Term Tobacco Smokers
Manuscript. [*)] *Contributed equally to this work.*

CONTENTS / INNEHÅLLSFÖRTECKNING

ABSTRACT **1**

RÉSUMÉ EN FRANÇAIS **3**

SVENSK SAMMANFATTNING **5**

INTRODUCTION **11**

Clinical importance of tobacco smoke 11

Tobacco smoke contents 11

Airway inflammation caused by tobacco smoke 12

Host defence 12
- Innate immunity 12
- Adaptive immunity 13

T cells in airway inflammation caused by tobacco smoke 13

Interleukin-16 14
- Protein 14
- Signalling 14
- Bioactivities 15
- Are there dual effects in inflammation? 15
- Involvement in disease 15

Pharmacological means to modulate IL-16 16
- Hydrocortisone 16
- Terbutaline 16
- Aminophylline 17
- Glutathione 17
- Cycloheximide 17
- Cyclosporine A 18
- Caspase-3 inhibitor 18

HYPOTHESIS **19**

AIMS **20**

General Aim 20
Specific Aims 20

METHODS **21**

Ethical considerations 21

Study population 21

Study groups for immunological analyses 21
Extracellular IL-16 protein in the airways of long-term tobacco smokers 21

Extra- and intracellular IL-16 protein in CD8$^+$ cells and IL-16 mRNA in the airways 22

Extracellular IL-16 protein in the airways from occasional tobacco smokers 22

IL-16 protein in human palatine tonsils 22

Extra- and intracellular IL-16 protein in CD8$^+$ cells in blood from long-term tobacco smokers 22

Definition of long-term tobacco smokers 23

Cell cultures 23
Isolation and culture of human CD8$^+$ cells 23
Preparation of Cigarette Smoke Extract 24
Pharmacological interventions 25

Assessment of IL-16 25
Assessment of extracellular IL-16 protein 25
Assessment of intracellular IL-16 protein 26
Assessment of IL-16 mRNA 26
Assessment of IL-16 protein in palatine tonsils 27

Assessment of protein oxidation 27

Assessment of viability 27

Data presentation and statistical analysis 28

Table 1 29

RESULTS **31**

Increased extracellular IL-16 protein in BAL from long-term tobacco smokers 31

No clear increase in extracellular IL-16 protein in BAL after short-term exposure to tobacco smoke 32

Tobacco smoke decreased IL-16 protein in palatine tonsils 33

CSE caused release of extracellular IL-16 protein from $CD8^+$ cells *in vitro* 34

Long-term tobacco smoking decreased intracellular IL-16 protein in BAL $CD8^+$ cells 34

Long-term tobacco smoking decreased IL-16 mRNA in BAL cells 36

Drugs without any clear effect on the CSE-induced increase of extracellular IL-16 protein in $CD8^+$ cells cultured *in vitro* 36

Glutathione and aminophylline concentration-dependently normalized the CSE-induced increase in extracellular IL-16 protein in $CD8^+$ cells cultured *in vitro* 37

Glutathione but not aminophylline normalized the CSE-induced decrease in intracellular IL-16 protein in $CD8^+$ cells *in vitro* 38

Glutathione normalized while aminophylline aggravated the CSE-induced decrease in intracellular IL-16 mRNA in $CD8^+$ cells *in vitro* 41

CSE increased oxidized proteins in $CD8^+$ cells *in vitro* 42

Long-term tobacco smoking did not result in a substantial change in extracellular IL-16 protein in blood 43

Long-term tobacco smoking did not result in a substantial change in intracellular IL-16 protein in blood $CD8^+$ cells 44

Summary of results *in vivo* 45

Summary of results *in vitro* 46

DISCUSSION **47**

Local impact of tobacco smoke on IL-16 protein and mRNA in $CD8^+$ cells in the lower airways 47

Systemic impact of tobacco smoke on extracellular IL-16 protein 48

Cellular source of IL-16 protein in the lower airways 48

Plausible mechanisms behind the release of IL-16 protein 50

Biological implications of the local increase in IL-16 protein in the lower airways 51

IL-16 and lung function 51

Modulating IL-16 in lung diseases 52

SUMMARY AND CONCLUSIONS **53**

TACK **55**

REFERENCES **58**

ABSTRACT

Background: There is an increased number of $CD8^+$ cells in the airways in chronic obstructive pulmonary disease (COPD) and also an increased number of $CD4^+$ cells in severe COPD. The CD4 cell chemoattractant interleukin (IL)-16 is also increased in the airways of tobacco smokers. In this thesis, we re-evaluated whether there is a local increase in IL-16 and determined whether there are systemic IL-16 alterations. We also investigated whether tobacco smoke causes a release of IL-16 in $CD8^+$ cells and elucidated cellular mechanisms.

Methods: We measured extracellular IL-16 protein (bronchoalveolar lavage fluid, BALF; plasma and serum), intracellular IL-16 protein (BAL $CD8^+$ cells) and IL-16 mRNA (BAL cells) in long-term tobacco smokers. In occasional tobacco smokers, we analysed extracellular IL-16 protein (BALF). IL-16 protein in tonsils of tobacco smokers was assessed. For the *in vitro* studies, isolated human blood $CD8^+$ cells were cultivated with and without water-soluble tobacco smoke components (CSE), an oxygen free radical (OFR) scavenger (glutathione) or a non-selective phosphodiesterase inhibitor (aminophylline) and analysed for extra- and intracellular IL-16 protein and IL-16 mRNA. Protein oxidation in CSE-treated $CD8^+$ cells was also measured.

Results: In long-term tobacco smokers, we confirmed an increase in IL-16 protein in BALF. We revealed a decrease in intracellular IL-16 protein in $CD8^+$ cells as well as in IL16 mRNA in BAL cells. We found no corresponding impact on IL-16 protein in plasma or serum. In contrast, occasional smokers did not exhibit any substantial alteration in IL-16 protein in BALF. However, tobacco smokers were found to have a decrease in IL-16 in tonsils. In cell culture of $CD8^+$ cells, CSE caused a release of IL-16 protein and a decrease in both

intracellular IL-16 protein and IL-16 mRNA. These alterations were prevented by glutathione but not by aminophylline. CSE-treated $CD8^+$ cells exhibited a marked increase in oxidized proteins.

Conclusion: Tobacco smoke mainly exerts an effect on IL-16 release locally in the airways. $CD8^+$ cells constitute a source of IL-16 and tobacco smoke depletes these cells by causing an extracellular release of this protein and a decrease in its mRNA. OFRs are involved as mediators of these effects.

RÉSUMÉ EN FRANÇAIS

Les effets de la fumée du tabac sur l'Interleukine 16, cytokine impliquée dans le recrutement des lymphocytes

Introduction: On observe un nombre accru de cellules $CD8^+$ dans les voies aériennes des patients souffrant de maladie pulmonaire obstructive chronique (MPOC) ainsi qu'un nombre accru de cellules $CD4^+$ dans les cas sévères de MPOC. L'interleukine chimiotactique de cellules CD4 (IL-16) est également présente en plus grand nombre dans les voies aériennes des fumeurs de tabac. Cette thèse vise à réévaluer si l'augmentation locale de l'interleukine-16 est effective et si les altérations de l'IL-16 sont systémiques. Des recherches ont également été menées pour déterminer si le tabac est à l'origine d'une libération de l'IL-16 dans les cellules $CD8^+$ et pour élucider les mécanismes cellulaires.

Méthodes: Nous avons mesuré la protéine IL-16 extracellulaire (liquide de lavage broncho-alvéolaire, BALF ; plasma et sérum), la protéine IL-16 intracellulaire (cellules $CD8^+$ BAL) et le niveau ARNm de l'IL-16 (cellules BAL) chez les fumeurs de tabac de longue durée. Chez les fumeurs occasionnels de tabac, nous avons analysé la protéine IL-16 extracellulaire (BALF). Il a également été procédé à l'évaluation de la protéine IL-16 dans les amygdales des fumeurs de tabac. Dans le cadre des études *in vitro*, des cellules $CD8^+$ isolées de sang humain ont été cultivées avec et sans les composants de la fumée de tabac soluble à l'eau (CSE), un radical libre d'oxygène (OFR), un piégeur (glutathione) ou un inhibiteur non-sélectif des phosphodiestérases (aminophylline) ; par ailleurs, des analyses ont été effectuées sur la protéine intracellulaire IL-16, la protéine extracellulaire IL-16 et l'ARNm de l'IL-16.

L'oxydation de la protéine dans les cellules $CD8^+$ traitées avec CSE a été mesurée.

Résultats: Chez les fumeurs de longue durée, nous avons confirmé une augmentation de la protéine IL-16 dans le BALF. Nous avons mis en évidence une diminution de la protéine intracellulaire IL-16 dans les cellules $CD8^+$ ainsi qu'une diminution de l'ARNm de l'IL-16 dans les cellules du BAL. Nous avons déterminé une absence d'impact sur la protéine IL-16 dans le plasma ou le sérum. À contrario, aucune altération substantielle de la protéine IL-16 dans le BALF n'est manifeste chez les fumeurs occasionnels. On observe toutefois une baisse de l'IL-16 dans les amygdales des fumeurs de tabac. En ce qui concerne la culture cellulaire des cellules $CD8^+$, CSE a engendré une libération de la protéine IL-16 et une diminution de la protéine IL-16 intracellulaire et du niveau ARNm de l'IL-16. Ces altérations ont été empêchées par le glutathione mais pas par l'aminophylline. Une augmentation significative des cellules $CD8^+$ traitées avec CSE a été observée dans les protéines oxydées.

Conclusion: La fumée de tabac exerce principalement un effet sur la libération de l'IL-16, de manière locale dans les voies aériennes. Les cellules $CD8^+$ constituent une source d'IL-16 et la fumée de tabac épuise ces cellules en provoquant une libération extracellulaire de cette protéine et une diminution du niveau ARNm. Les OFR interviennent en tant que médiateurs de ces effets.

SVENSK SAMMANFATTNING

Tobaksrökens effekter på den lymfocytrekryterande cytokinen interleukin-16

Tobaksrökning och luftvägssjukdom

Tobaksrökning medför bland annat inflammation i lungorna som drabbar både luftvägsträdet (bronkerna) samt lungblåsorna (alveolerna). I luftvägarna leds luften ner till lungblåsorna, där luftens syrgas tas upp och koldioxiden från arbetande celler avges för att sedan forslas bort med utandningsluften. Inflammationen som orsakas av tobaksrökning kan leda till att lungorna oåterkalleligen skadas och vid svår lungskada har man utvecklat kroniskt obstruktiv lungsjukdom (KOL). Denna sjukdom drabbar främst rökare och omfattar alltid luftvägarna och oftast lungblåsorna. Vid KOL är lungfunktionen nedsatt och detta kan leda till andningshandikapp.

Ospecifikt och specifikt immunförsvar

Kroppens immunförsvar kan översiktligt delas upp i ett ospecifikt och i ett specifikt immunförsvar. När främmande och skadliga gaser eller luftburna ämnen, bakterier eller virus (mikrober) kommer ner i lungorna aktiveras det ospecifika immunförsvaret. Genom olika mekanismer kan då försvarscellerna avge substanser som oskadliggör det sjukdomsalstrande ämnet eller mikroben. Det specifika immunförsvaret lär sig känna igen enskilda mikrober och kan döda dem med hjälp av bland annat antikroppar som bildas av B-celler. Både B- och T-celler är viktiga medarbetare i det specifika immunförsvaret tillhörande gruppen vita blodkroppar (leukocyter). T-cellerna kan delas upp i hjälparceller ($CD4^+$ celler), som är viktiga för att ”dirigera och leda” immunförsvaret och cytotoxiska celler ($CD8^+$ celler), vilka känner igen en infekterad cell och dödar den.

Tobaksrökning och antalet $CD4^+$ och $CD8^+$ celler i lungorna

I lungorna hos rökare har tidigare forskning visat på en ökning av antalet $CD8^+$ celler, liksom att antalet $CD8^+$ celler ökar ju mer uttalad lungfunktions-nedsättningen är. Hos rökare med KOL, åtminstone hos dem med svår KOL, finns även en ökning av antalet $CD4^+$ celler i lungorna enligt flera tidigare studier.

Protein och mRNA

Äggviteämnen (proteiner) tillverkas av kroppen i stor mängd. Dessa är sinsemellan strukturellt olika eftersom deras funktion skiljer sig åt. Proteiner kan utgöra alltifrån byggelement i muskler till cytokiner (såsom interleukin-16, förkortat IL-16). IL-16 framställs efter en genetiskt bestämd ”mall” kallat IL-16 mRNA, i likhet med andra proteiner som görs efter sitt särskilda mRNA. Detta mRNA finns inuti cellen (intracellulärt) och bestäms således av den genetiska information som bärs av cellkärnans DNA.

Cytokiner och interleukiner

Cytokiner är den övergripande beteckningen för de substanser som kan bildas och avges från alla de celler i kroppen som bidrar till immunförsvaret. För cytokiner som produceras av vita blodkroppar brukar beteckningen interleukiner (”inter”=mellan och ”leukiner”=vita blodkroppar) användas. Cytokinernas funktion är att signalera och överföra informationen till andra celler.

Bronkoskopi

Med hjälp av en tunn slang innehållande optiska fibrer som förs ner i luftvägarna (bronkoskopi) kan man se hur det ser ut nere i luftvägarna och ta prover. Ett vanligt sätt att få biologiskt provmaterial är att skölja nere i luftvägarna med en mindre mängd koksaltlösning och samla in utbytet (BAL-prov; BAL står för bronchoalveolar lavage). Bronkoskopet förs då ned till den delen av luftvägarna varifrån provtagning önskas. Man sköljer sedan med koksaltlösningen som sedan sugs upp igen. I detta BAL-prov får man då både med sig celler samt lösliga substanser (såsom proteiner) vilka sedan kan mätas.

Interleukin-16 och dess funktion

I en tidigare studie från vår forskargrupp visades att det i BAL-prov från tobaksrökare finns en ökning av IL-16 protein jämfört med icke-rökare. IL-16 kan bland annat bildas och avges från $CD8^+$ celler. Dock var det inte känt när det aktuella avhandlingsarbetet påbörjades om tobaksrök kunde orsaka denna frisättning av IL-16. En viktig funktion hos IL-16 synes vara att den lockar till sig $CD4^+$ celler genom så kallad kemotaxi. Kemotaxi innebär här att $CD4^+$ celler (och i viss mån andra celler) vandrar mot en koncentrationsgradient av IL-16 och ansamlas där dess koncentration är som högst.

Frågeställning

Det främsta målet med detta avhandlingsarbete är att öka kunskapen om och förståelsen för samspelet mellan tobaksrökning, IL-16 och $CD8^+$ celler, eftersom det finns goda grunder att tro att detta samspel kan vara en del i den inflammation som leder till KOL.

Mätning av IL-16 från luftvägar, tonsiller och blod

Till att börja med kunde vi i ett nytt patientmaterial genom BAL-prov hos rökare bekräfta fyndet att IL-16 proteinnivån är ökad i lungorna jämfört med icke-rökare. Vi fann också i BAL-prov att den intracellulära mängden av IL-16 är sänkt i $CD8^{+}$ celler hos tobaksrökare. Vidare mätte vi nivån av IL-16mRNA från BAL-provsceller och fann att även den är sänkt hos tobaksrökare.
För att undersöka om tobaksrökens effekter på IL-16 är ett lokalt eller systemiskt fenomen skattades mängden IL-16 i tonsiller som hade opererats bort av medicinska skäl. Vi såg i detta fall en minskning av IL-16 i tonsiller hos tobaksrökare jämfört med icke-rökare, vilket är förenligt med en systemisk påverkan av tobaksröken på IL-16. Av den anledningen mätte vi även IL-16 protein i blod från tre grupper av individer. Vi undersökte friska personer som aldrig hade rökt och asymptomatiska rökare, båda dessa grupper hade en normal lungfunktion samt KOL-sjuka med nedsatt lungfunktion. Våra resultat visar att ingen betydande skillnad av IL-16 koncentrationen finns i blodet mellan dessa grupper. Sammantaget talar detta för att tobaksrökens effekter på fritt och lösligt IL-16 utanför cellerna huvudsakligen sker lokalt i luftvägarna.

Mätning av IL-16 från cellodlingar

$CD8^{+}$ celler från anonyma blodgivare från blodcentralen renades fram. Dessa $CD8^{+}$ celler från blod odlades med och utan vattenlösligt tobaksrökextrakt (CSE). Härigenom kunde vi ta reda på om CSE medför IL-16 frisättning från $CD8^{+}$ celler samt få information om mekanismerna bakom och om en sådan frisättning kunde blockeras med läkemedel.
Mängden av fritt och lösligt IL-16 som avgivits till cellodlingsvätskan mättes, likaså mängden av IL-16 protein intracellulärt och IL-16mRNA nivån i cellerna. Vi fann att CSE medför att $CD8^{+}$ cellerna frisätter IL-16 protein. Vidare såg vi att CSE minskar både mängden av IL-16 protein intracellulärt samt även nivån av IL-16 mRNA i cellerna.

I nästa steg undersökte vi om läkemedel kunde påverka effekten av CSE på IL-16 protein som avgivits till odlingsvätskan, mängden av IL-16 protein intracellulärt samt nivån av IL-16mRNA i cellerna. Vi använde oss bland annat av glutathion och aminofyllin, vilka är substanser som utnyttjas vid behandling av inflammatoriska luftvägssjukdomar. Glutathion respektive aminofyllin tillsattes till ovan nämnda försök på renframställda $CD8^+$ celler som behandlades med CSE. Vi noterade att frisatt mängd IL-16 till odlingsvätskan minskade och normaliserades med ökad dos av nämnda läkemedel. Intressant nog medförde även glutathion en normalisering av mängden IL-16 protein intracellulärt samt även av IL-16mRNA i cellerna. I motsats till denna normalisering som skedde med tillsats av glutathion, så ledde aminofyllin-behandling till en än kraftigare sänkning av IL-16mRNA i cellerna men ingen ändring av IL-16 intracellulärt, jämfört med om cellerna enbart hade odlats med CSE.

Sammanfattande tolkning av fynden

Vi såg en stor överensstämmelse mellan fynden från luftvägarna hos tobaksrökare och från cellodlingarna. Härigenom kunde vi dra följande slutsatser:

- Tobaksök medför frisättning av IL-16 från $CD8^+$ celler som sålunda är en tänkbar källa till den förhöjda nivån av IL-16 protein som återfinns i luftvägarna hos rökare.
- Långvarig tobaksrökning inverkar menligt på $CD8^+$ cellerna och produktionen av IL-16, sannolikt via fria syreradikaler.
- Glutathion upphäver tobaksrökens skadliga effekter, i vart fall hos renframställda $CD8^+$ celler.
- Tobaksrökens påverkan på fritt och lösligt IL-16 utanför cellerna sker till största delen lokalt i luftvägarna och inte systemiskt ute i kroppen.

Vad har fynden för betydelse?

Den ökade nivån av IL-16 i luftvägarna hos tobaksrökare som har rökt mycket och länge kan tänkas utgöra ett mål för läkemedelsbehandling mot luftvägsinflammation orsakad av tobaksrök; där glutathion som hämmar fria syreradikaler utgör ett exempel på en lovande behandlingsprincip. Teoretiskt sett skulle IL-16 bidra till att antalet $CD4^+$ celler ökar i lungorna vid KOL men vi vet ännu inte om denna ansamling av $CD4^+$ celler är till nytta eller till skada. För att pröva den kliniska nyttan med en behandlingsprincip som påverkar IL-16 krävs nya studier på KOL-patienter.

INTRODUCTION

Clinical importance of tobacco smoke

Tobacco smoking is a major risk factor for a number of clinically important disorders, including chronic obstructive pulmonary disease (COPD) [1, 2]. Worldwide, COPD is the fourth leading cause of mortality, predicted to become the third in 2020 [3, 4]. In principle, the *pathophysiological* definition of COPD is airflow limitation that is not fully reversible after treatment with a bronchodilator [2]. The chronic airflow limitation is caused by small airway disease, which mainly affects bronchioles in the peripheral airways with an internal diameter < 2 mm (obstructive bronchiolitis) and lung parenchyma with destruction of the respiratory bronchioles and alveoli (emphysema) [2]. Another clinically important condition related to tobacco smoke is chronic bronchitis (CB), which is relatively common among patients with COPD [1]. CB is defined by the *clinical history* obtained from the patient, requiring production of sputum for ≥ 3 months in two consecutive years, not always associated with significant airflow limitation [2]. Notably, some subjects with COPD display extrapulmonary manifestations of the disease such as ischemic heart disease and osteoporosis [5, 6].

Tobacco smoke contents

Tobacco smoke from cigarettes contains a substantial number of components. More than 5000 compounds have been identified, but the pathogenic potential of most of them remains unclear [7, 8]. Nicotine, hydroquinone and nitrosamines are some of the detrimental components that have been examined [9-11]. Tobacco smoke also contains other bioactive compounds, such as endotoxin and oxidants, i.e. free radicals that cause oxidative stress [7, 12].

Airway inflammation caused by tobacco smoke

The airway inflammation caused by tobacco smoke is characterized by an increase in the number of immune cells. Traditionally, neutrophils, macrophages and $CD8^+$ (cytotoxic T) cells have been associated with the pathogenesis of COPD [8, 13]. Recent studies have revealed that $CD4^+$ (T helper cells) and B cells are increased in tobacco smoke induced airway inflammation and thus may contribute to the disease [8, 14-16].

Host defence

Innate immunity. Mammals including humans have a rapid and non-specific component of host defence that contributes to protection from infections; this component is termed innate immunity. In evolutionary terms, this is an older defence system than adaptive immunity and includes the complement system, antimicrobial compounds and oxidative stress [17, 18]. Neutrophils and macrophages are effector cells of particular importance in innate immunity, since they are able to kill bacterial pathogens in a non-specific manner [19]. NK (natural killer) cells are mainly regarded as belonging to the innate immunity. They recognize virally infected or tumour cells and kill them [19].

One aspect of innate immunity is its capacity to generate reactive oxygen species (ROS) and thus, oxidative stress, which is strongly linked to inflammation and has both endogenous and exogenous origins. Among ROS, hydroxide peroxide and hydroxyl radicals can cause damage via free radicals. Epithelial cells, macrophages and neutrophils are examples of cells that can produce ROS upon inflammatory stimulation. In this way, ROS constitute an important defence system against bacterial infections. It has been shown that ROS reduce the synthesis of elastin and collagen, inactivate antiproteases and activate the transcription factor NF- κβ, which activates inflammatory genes such as the gene of TNF-α [12, 20]. In both animal models and humans,

acetylcysteine, the precursor of glutathione, exerts antioxidant effects that neutralize those of ROS [12].

Adaptive immunity. This highly sophisticated defence system is unique to vertebrates [17]. In order to recognize and eliminate a specific pathogen, evolution has developed the adaptive immunity that comprises specific receptors. The most important effector cells are B and T cells. The B cells produce antibodies in response to antigen stimulation to combat infections. In the same way as B cells, T cells have a unique antigen receptor, which recognizes intruder structures. The T cells can be divided into two subgroups, $CD4^+$ and $CD8^+$ cells. Of these subgroups, $CD4^+$ cells provide signals for activating other immune cells such as macrophages, neutrophils and B cells [19, 21]. $CD8^+$ cells are capable of killing virally infected cells. In contrast to innate immunity, both B and T cells generate memory cells after an infection, which will result in a more rapid and stronger immunological response when encountering the same pathogen on a future occasion [19].

T cells in airway inflammation caused by tobacco smoke

Several studies confirm that T cells are increased in the airways of tobacco smokers and likely to be actively involved in the inflammatory process. In particular, their ability to produce cytokines that recruit monocytes and neutrophils illustrates the close link between adaptive and innate immunity [21, 22]. In 1995, Finkelstein *et al.* found an inverse correlation between the degree of emphysema in the lungs of smokers and the number of T cells [23]. Subsequent studies established a correlation between an increase in $CD8^+$ cells in the airways and decline in lung function in tobacco smokers [24, 25]. More recent studies have demonstrated that $CD4^+$ cells are increased in the airways of tobacco smokers, not least in those with COPD [14-16].

Interleukin-16

Protein. Interleukin-16 (IL-16) was first identified in 1982 as a lymphocyte chemoattractant factor (LCF) in T cells [26, 27]. From an evolutionary perspective, IL-16 protein is highly conserved and both simian and murine IL-16 protein exert the same biological activity as human IL-16 on human T cells [28]. In 1985, it was reported that both CD8$^+$ and CD4$^+$ cells produce IL-16 but that the protein exhibits selective and potent chemoattractant activity only in CD4$^+$ cells [29]. In humans, the IL-16 gene is located on chromosome 15. IL-16 is generated as pro-IL-16, with a 631 amino-acid precursor and a molecular mass of around 68 kDa. Pro-IL-16 is cleaved by active caspase-3 into IL-16, which consists of a 121 amino-acid molecule with a weight of about 17 kDa [28, 30, 31]. Bioactivity only occurs after auto-aggregation of IL-16 in dimers or tetramers [28]. IL-16 can be released upon stimulation of cells*,* although the release mechanism is not fully understood [28, 32]. The exact mechanism behind the production of IL-16 varies for different cells. Unstimulated CD8$^+$ cells contain bioactive IL-16 protein, in contrast to CD4$^+$ cells which only have pro-IL-16 [28, 33]. The explanation is that resting CD8$^+$ cells contain active caspase-3, which cleaves pro-IL-16, resulting in a pool of preformed, bioactive IL-16. Resting CD4$^+$ cells only contain pro-caspase-3. When these cells are activated after stimulation, cleavage of pro-caspase-3 into active caspase-3 occurs, resulting in the production of bioactive IL-16 [30].

Signalling. Surface expression of the CD4 receptor is required for IL-16 bioactivities [28] . Thus, cells bearing the CD4 receptor will respond to IL-16 protein. It is believed that cross-linking of the CD4 receptors is necessary, since only the multimeric forms (dimers and tetramers) of IL-16 induce bioactivity, not the monomer [28]. This activation of the CD4 receptor results in phosphorylation of STAT 6, a protein in the cytoplasm, which then translocates into the nucleus and exerts its effects by activating or repressing target genes [34].

Bioactivities. IL-16 protein is a chemoattractant, inducing cell migration towards a concentration gradient [28]. Intrapleural injection of recombinant human (rh) IL-16 protein into the pleural space of subjects with pleural effusions results in a significant increase in $CD4^+$ cells observed after 12 h and reaching a maximum at 24 h [35]. IL-16 protein is also a growth factor. When $CD4^+$ cells are cultivated with both IL-16 and IL-2 *in vitro*, they proliferate markedly. In contrast, if only IL-2 is added to the $CD4^+$ cells, there is merely a slight proliferation [36].

Are there dual effects in inflammation? Some studies designate IL-16 as a pro-inflammatory cytokine. The secretion of the pro-inflammatory cytokines IL-1β and TNF-α was seen when rhIL-16 was cultivated *in vitro* with human monocytes [37]. In contrast, others consider IL-16 an anti-inflammatory cytokine because treatment with rhIL-16 in a mouse model of human synovial tissue from subjects with rheumatoid arthritis resulted in a decrease in IL-1β and TNF-α, measured as mRNA [38]. In mouse models of encephalomyelitis and renal ischemia-reperfusion injury, respectively, anti-IL-16 treatment blocked the influx of $CD4^+$ cells and improved the outcome [39, 40].

Involvement in disease. Previous studies have identified alterations in IL-16 in a number of diseases, even if it is not clear whether these changes are pathogenically important. Some studies have linked IL-16 to asthma and T cells. Subjects with atopic asthma exhibit an increase in IL-16 immunoreactivity (IR) in bronchial mucosa, which correlates strongly in a positive manner with the number of $CD4^+$ cells in the same compartment [41]. Extracellular IL-16 protein as well as chemoattractant capacity of T cells have been found in bronchoalveolar lavage (BAL) fluid after allergen challenge in asthmatics [42, 43]. A study of lung allograft recipients showed that subjects with acute rejection lacked the increase in IL-16 protein in BAL fluid observed

in those who did not develop rejection [44]. Other studies have revealed that the concentration of IL-16 protein is increased in serum as well as in plasma, in multiple myeloma, rheumatoid arthritis, systemic lupus erythematosus and after traumatic injury [45-48]. Regarding tobacco smoke and IL-16, our research group has published evidence prior to the present work that the concentration of IL-16 protein in BAL fluid is higher in tobacco smokers than in never-smokers. Our previous study also demonstrated a negative correlation between the concentration of IL-16 in the airways and the number of peripheral blood $CD4^+$ cells [49].

Pharmacological means to modulate IL-16

Hydrocortisone. To date, four steroid hormone receptors have been identified; the glucocorticoid receptor (binding hydrocortisone, also called cortisol), the mineralocorticoid receptor (binding aldosterone), the progesterone receptor (binding progesterone) and finally, the androgen receptor (binding testosterone) [50]. The anti-inflammatory effects of hydrocortisone are exerted in many ways, for example by increased transcription of anti-inflammatory genes such as Iκβ-α (NF-κβ inhibitor) and decreased transcription of TNF-α [51]. In COPD treatment, inhaled corticosteroids seem initially to slightly improve lung function, measured as forced expiratory volume in 1 second (FEV_1) but do not modify the long-term decline in FEV_1 [52]. However, inhaled glucocorticoids plus long-acting β_2 agonists reduce the number of exacerbations [52].

Terbutaline. Terbutaline is a selective and short-acting β_2 agonist. The β2-receptors are widely distributed in the lung tissue and stimulation of the receptors results in smooth airway relaxation [53]. Terbutaline is thus a bronchodilator. Furthermore, *in vitro* studies indicate that β2- agonists inhibit T cell proliferation [54]. As mentioned above, in COPD, long-acting β_2 agonists

are often used in combination with inhaled glucocorticoids to obtain a synergistic effect, thus reducing the number of exacerbations [52].

Aminophylline. Theophylline and aminophylline are both non-selective phosphodiesterase (PDE) inhibitors belonging to the methylxanthines, which are related to caffeine and have been utilized in the treatment of obstructive airway diseases such as COPD over a long period of time. Aminophylline contains the ethylenediamine salt of theophylline and was used in our study because it is more water-soluble and therefore suitable for *in vitro* cultures [55-57]. The inhibition of PDE results in an intracellular increase in cAMP, leading to relaxation of airway smooth muscle with bronchodilatation. Theophylline also prevents the transcription factor NF-κβ from reaching the nucleus, thus diminishing the transcription of several inflammatory cytokines [57]. Theophylline is still used for bronchodilator treatment, especially when the effect of other drugs is insufficient [52].

Gluthathione. This antioxidant is present in high concentrations in the epithelial lining fluid of the lower airways and important for protecting against harmful agents caused by oxidative stress but its level is increased in smokers [58]. Acetylcysteine, a precursor of glutathione, is commonly employed as a mucolytic agent for the prevention of exacerbation in COPD [12, 52]. However, one published study revealed that acetylcysteine did not reduce the number of exacerbations, with the exception of a subgroup with no inhaled corticosteroids [59].

Cycloheximide. Cycloheximide is a drug that acts by inhibiting the translation of the proteins, i.e., blocking the step between mRNA and the protein synthesis [60]. Cycloheximide is only used in biomedical research and not in humans due to its toxic side effects.

Cyclosporine A. This drug acts selectively on T cells and was initially isolated from a soil fungus. Cyclosporine A is used in the treatment of diseases such as rheumatoid arthritis and psoriasis as well as to prevent rejection in transplant patients. It has no established place in the treatment of COPD but has been tested in asthma [61, 62]. Cyclosporine A inhibits intracellular calcineurin phosphatase, thus blocking the transcription factor NFAT by preventing the transcription of a number of genes encoding for several cytokines [62].

Caspase-3 inhibitor. The caspases constitute a family of enzymes, many of which have been linked to apoptosis [63]. Caspase-3 cleaves pro-IL-16 into bioactive IL-16 which exhibits chemoattractant ability in contrast to pro-IL-16 [31]. In $CD8^+$ cells, caspase-3 is constitutively present, resulting in preformed, bioactive IL-16 [30]. In contrast, $CD4^+$ cells require stimulation for the production of bioactive IL-16***,*** which depends on these cells in resting condition only containing pro-caspase-3, which is not enzymatically active [30]. However, studies have shown that IL-16 release can occur without any subsequent cell apoptosis [28, 64]. This type of drug is not used clinically.

HYPOTHESIS

In the present thesis, it was hypothesised that tobacco smoke affects the production and release of IL-16 protein in T cells, thus leading to an altered expression of IL-16 in the airways and blood.

AIMS

General Aim

The general aim of this thesis was to determine whether and how tobacco smoke affects the production and release of interleukin-16 in humans, especially the relation of this protein to $CD8^+$ cells.

Specific Aims

1. To re-evaluate whether long-term tobacco smokers have increased extracellular IL-16 protein in the airways and, if so, to investigate whether it can be caused by short-term exposure to tobacco smoke and whether long-term exposure exerts an impact on IL-16 protein in lymphoid tissue (**Papers 1-2**).

2. To determine whether $CD8^+$ cells can release extracellular IL-16 protein upon stimulation with tobacco-smoke components *in vitro* and thus constitute a source of this protein in tobacco smokers (**Paper 1**).

3. To determine whether tobacco smoke alters the IL-16 biology in $CD8^+$ cells both *in vivo* and *in vitro* with respect to the content of intracellular IL-16 protein and IL-16 mRNA transcription (**Paper 2**).

4. To determine whether the release of extracellular IL-16 protein, depletion of intracellular IL-16 protein and decrease in IL-16 mRNA transcription caused by tobacco-smoke components can be prevented by pharmacological means *in vitro* and whether oxygen free radicals are likely to be involved (**Paper 2**).

5. To determine whether long-term tobacco smokers with and without COPD have systemic alterations in blood IL-16 (**Paper 3**).

METHODS

Ethical considerations (Papers 1-3)

The studies were approved by the Ethics Committee at the University of Gothenburg. Oral and written consent was obtained from the participants.

Study population (Papers 1-3)

For this thesis, material was obtained from 5 different human populations as described below and in Table 1 (pp. 29-30).

Study groups for immunological analyses

Extracellular IL-16 protein in the airways of long-term tobacco smokers (Paper 1). Bronchoscopy was performed in a study population and BAL (bronchoalveolar lavage) was harvested for analysis of extracellular IL-16 protein in the airways. The study population consisted of current long-term tobacco smokers with a tobacco load corresponding to ≥ 10 pack-years with and without chronic bronchitis (CB), respectively. The latter group was termed asymptomatic smokers (AS). The control group was composed of never-smokers (NS). Both the NS and AS subjects were required to have normal lung function defined as FEV_1 > 80% of predicted (% pred.). In contrast, co-existing airflow obstruction was allowed in the CB group defined as FEV_1 <80 (% pred.). See Table 1. The CB group was required to have ≥ 2 acute exacerbations during the previous year.

Extra- and intracellular IL-16 protein in CD8+ cells and IL-16 mRNA in the airways (Paper 2). BAL samples were harvested from long-term tobacco smokers with current smoking corresponding to ≥ 10 pack-years and from a control group consisting of never-smokers. Bronchoscopy was performed on a clinical basis and the subjects were not allowed to exhibit any signs of infection 4 weeks prior to the investigation. Both FEV_1 (% pred.) and the ratio of FEV_1 and forced vital capacity (FVC) were calculated for the two groups (Table 1).

Extracellular IL-16 protein in the airways from occasional tobacco smokers (Paper 2). This group was composed of occasional smokers with normal lung function defined as normal FEV_1 (% pred.) and a normal FEV_1/FVC quota, smoking 1-4 times a month but not during the 4 weeks prior to the study (Table 1). Never-smokers were included as a control group. BAL samples were harvested on days 1 and 14 and the occasional smokers were requested to smoke a total of 10 cigarettes on days 12 and 13.

IL-16 protein in human palatine tonsils (Paper 1). The tonsils were obtained from subjects undergoing routine tonsillectomy because of hypertrophy and chronic tonsillitis. All smokers were currently smoking, with a tobacco load of ≥ 3 pack years. The control group was composed of non-smokers, of whom one subject had stopped smoking > 9 years earlier (Table 1).

Extra- and intracellular IL-16 protein in CD8+ cells in blood from long-term tobacco smokers (Paper 3). Three different groups were included in the study: smokers with COPD (GOLD stages 2 and 3), asymptomatic smokers (AS) and never-smokers (NS). All smokers were current and long-term smokers with a tobacco load of > 10 pack-years. For inclusion, the smokers with COPD were required to have $FEV_1/FVC < 0.7$. For

subjects over 65 years, a ratio of $FEV_1/FVC < 0.65$ was regarded as abnormal [52]. Furthermore, the diffusion capacity for carbon monoxide (DL_{CO}) had to be reduced by > 2 standard deviations (SD) from the predicted mean value in the group of smokers with COPD and served as a marker of emphysema. In contrast, both AS and NS exhibited normal spirometry in addition to normal DL_{CO} (Table1). A peripheral venous blood sample was drawn for analysis of IL-16 protein in plasma*,* serum and for flow cytometry analysis of leukocytes, respectively.

Definition of long-term tobacco smokers

For analysis of IL-16 protein in the airways, the smokers in Paper 1, defined as asymptomatic smokers (AS) and those with chronic bronchitis (CB), had a tobacco load of ≥ 10 pack-years and were termed long-term tobacco smokers. The smokers in Paper 2, described as chronic tobacco smokers, in whom we assessed intracellular IL-16 protein in $CD8^+$ cells as well as IL-16 mRNA in BAL, were also defined as long-term tobacco smokers when they had a tobacco load of ≥ 10 pack-years. This applied to all smokers in Paper 3 for the analysis of IL-16 in blood. In contrast, the subjects for analysis of IL-16 protein in tonsils exhibited a shorter average exposure to tobacco smoke. Due to this smaller tobacco load, they were not defined as long-term tobacco smokers. The explanation for the smaller tobacco load among this group is due to the fact that subjects undergoing routine tonsillectomy are younger.

Cell cultures

Isolation and culture of human $CD8^+$ cells (Papers 1-2). Human $CD8^+$ cells were prepared from buffy coat from clinically healthy blood donors. The $CD8^+$ cells were isolated by density gradient centrifugation followed by negative depletion with magnetic antibodies. The isolated $CD8^+$ cells were then

cultured with and without stimulation by the water-soluble components of cigarette smoke extract (CSE) as well as with CSE plus the respective drug. The complete medium alone served as a negative control (Papers 1-2) as did the complete medium including the highest utilised drug concentration without CSE (Paper 2). Stimulation with calcium ionophore (CI) and phorbol 12-myristate 13-acetate (PMA) was utilised as positive control conditions. The $CD8^+$ cells were cultured for 10 hrs (IL-16 mRNA) or 20 hrs (extra- and intracellular IL-16 protein and measurements of protein oxidation).

Preparation of Cigarette Smoke Extract (Papers 1-2). The water-soluble components of tobacco smoke were extracted by drawing the generated tobacco smoke from commercially available cigarettes (Marlboro) by means of vacuum through 15 ml of cell culture medium (RPMI-1640). The solution was thereafter filtered in a sterile manner, divided into aliquots and frozen (-80°C) for storage. The concentration of CSE chosen for experimental use generated reproducible responses with reference to IL-16, without a detectable detrimental effect on cell viability. No endotoxin was detected in the CSE batches. (Figure 1).

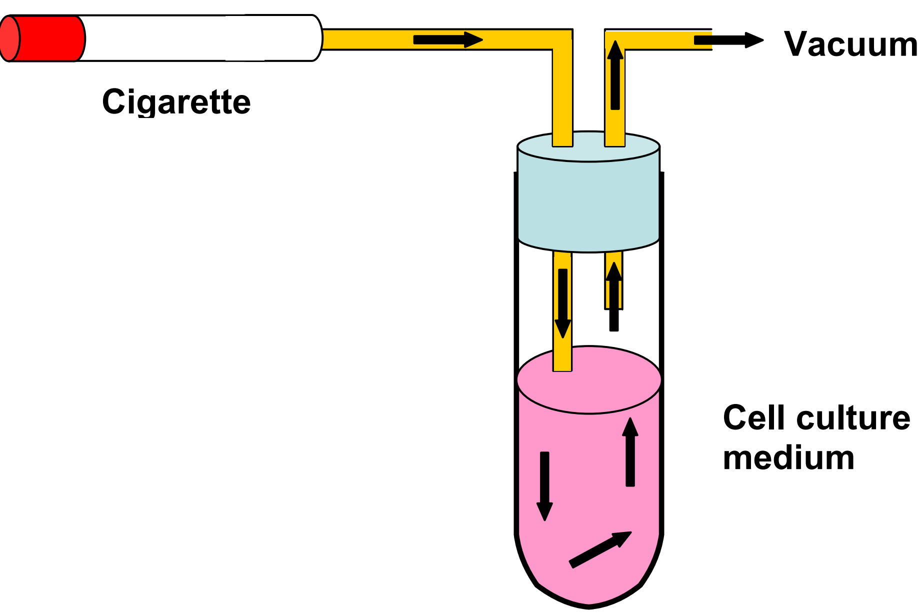


Figure 1. *Preparation of the water-soluble tobacco smoke components (CSE).*

Pharmacological interventions (Paper 2). This step was undertaken in order to determine whether CSE-induced alterations in IL-16 biology can be prevented by pharmacological means and to characterize cellular CSE exposure mechanisms. We tested several clinically utilised COPD drugs, including a non-selective phosphodiesterase inhibitor (aminophylline), a glucocorticoid receptor agonist (hydrocortisone) and a beta-adrenoceptor agonist (terbutaline). We also tested co-treatment with hydrocortisone and terbutaline. To further elucidate cellular mechanisms, a protein synthesis inhibitor (cycloheximide), a calcineurin phosphatase inhibitor (cyclosporine A) and a caspase-3 inhibitor, respectively, were also used. In addition, we examined an oxygen free radical scavenger (OFRs); glutathione). The experiments on CSE-induced release of extracellular IL-16 protein were performed with the drugs described above. Only glutathione and aminophylline exerted a clear and reproducible impact on this release. For this reason, the following studies on intracellular IL-16 protein and IL-16 mRNA were carried out with glutathione and aminophylline.

Assessment of IL-16

Assessment of extracellular IL-16 protein (Papers 1-3). The concentration of IL-16 protein was measured using commercially available ELISA kits. The BAL samples (**Papers 1-2**) were concentrated ≥ 10-fold before measurement of the IL-16 protein. The presented values were corrected for this process. In blood (plasma and serum; **Paper 3**) and in the experiments carried out *in vitro* (**Papers 1-2**), the IL-16 analyses were performed in un-concentrated samples.

Assessment of intracellular IL-16 protein (Papers 2-3). To study the intracellular concentration of IL-16 protein in $CD8^+$ cells, flow cytometry analyses were performed. For the analysis of intracellular IL-16 protein in BAL $CD8^+$ cells (**Paper 2**) and in peripheral blood $CD8^+$ cells (**Paper 3**), a protein transport inhibitor (GolgiStop) was added immediately after harvest of the cells to optimize the detection of the protein. However, in the CSE-treated and purified $CD8^+$ cells cultured for 20 hrs in vitro, the protein transport inhibitor was added 6 hrs prior to harvest (**Paper 2**). In order to identify $CD8^+$ cells harvested in BAL, we used antibodies (Abs) for the surface markers CD45 and CD8. In addition, to ensure healthy cells we excluded those staining positive for the cell integrity marker 7-AAD. For the detection of $CD8^+$ cells in blood, we used Abs for the surface markers CD45 and CD8. In the *in vitro* experiments, the cultured $CD8^+$ cells were detected by CD8 Abs alone. In the next step, the cell membranes were permeabilized with saponin buffer, in order to facilitate the IL-16 Abs to reach and stain the intracellular IL-16 protein. Finally, cellular analysis was performed using a flow cytometer. The relative number of IL-16 positive $CD8^+$ cells and the intensity of the intracellular staining (i.e. the concentration of IL-16 protein), rMFI (relative Mean Fluorescence Index), were calculated.

Assessment of IL-16 mRNA (Paper 2). An RNA-stabilizing solution was added immediately after harvest of BAL cells or after culturing $CD8^+$ cells for 10 hrs (RNA-later). RNA purification was then performed using an RNeasy Mini Kit, after which complementary DNA (cDNA) was generated by means of reverse transcriptase. After amplification of cDNA, real-time RT–PCR was carried out. The IL-16 mRNA expression was normalised to the housekeeping gene HPRT.

Assessment of IL-16 protein in palatine tonsils (Paper 1). The sections of human palatine tonsils were stained with IL-16 Abs after permeabilization with saponin. The immune reactivity (IR) was assessed in a blinded manner using light microscopy. The result was expressed as area percentage of immune staining.

Assessment of protein oxidation (Paper 2)

To characterize the degree of protein oxidation caused by tobacco smoke components, i.e. the involvement of OFR and oxidative stress, the $CD8^+$ cells were cultivated for 20 hrs with and without CSE. This method is based upon the fact that, as a consequence of oxidative stress, carbonyl groups are introduced into proteins. Briefly, the harvested $CD8^+$ cells were lysed followed by Western blot for the detection and quantification of the oxidized proteins using a commercially available kit. The protein carbonyl groups must be derivatized with DNPH (2,4-dinitrophenylhydrazine) before they can be detected. The final detection and quantification step was then performed using chemiluminescence.

Assessment of viability (Papers 1-2)

Viability was assessed using exclusion of trypan blue dye. In short, if the integrity of the cell membrane was damaged, the trypan blue dye entered intracellular space and coloured the cell blue. The quantification was performed using a light microscope.

Data presentation and statistical analysis

Non-parametric statistics were employed with one exception (see below). Differences were considered statistically significant for p-values < 0.05. For comparison between multiple groups, the Kruskal-Wallis test was used followed by the Mann-Whitney U test (Papers 1-3). For comparison between two groups, the Mann-Whitney U test (Papers 1-3) and Wilcoxon´s signed rank test (Paper 1) were applied. The correlation analyses were performed using the Spearman Rank Correlation test (Papers 2-3). The paired t-test was applied for the analysis of protein oxidation (Paper 3). Data are presented as individual plus median values.

Table 1. Demographic and clinical data on participating subjects in the thesis

	Never-smokers	Asympt.-smokers	Smokers with CB	Non-smokers	Smokers	Never-smokers	Chr. tob. smokers
Paper no.	I	I	I	I	I	II	II
n	7	8	33	6	6	5 **	7 **
Compartment	BAL	BAL	BAL	Tonsil	Tonsil	BAL	BAL
Target	IL-16 EC	IL-16 EC	IL-16 EC	IL-16	IL-16	IL-16 IC	IL-16 IC
Age (years)	51 (37-64)	45 (34-60)	50 (37-68)	30 (17-49)	28 (21-55)	64 (20-79)	61 (26-73)
Pack-years	0	25 (11-62)	34 (12-88)	0 (0-4)*	10 (3-15)	0	31 (10-49)
Male/female	4/3	3/5	6/27	3/3	3/3	3/2	5/2
FEV_1 (% pred.)	105 (91-120)	108 (95-129)	83 (61-111)	x	x	113 (89-128)	95 (34-100)
FEV_1 / FVC	x	x	x	x	x	77 (71-96)	69 (39-76)
DLco (% pred.)	x	x	x	x	x	x	x

*) One non-smoker stopped smoking > 9 years prior to the study.

**) Some of the subjects are included in the analyses of both rMFI and mRNA.

EC=extracellular. IC=intracellular. Chr. tob. smokers=chronic tobacco smokers

Table 1 cont. Demographic and clinical data on participating subjects in the thesis

	Never-smokers	Chr. tob. smokers	Never-smokers	Occasion. smokers	Never-smokers	Asympt.-smokers	Smokers with COPD
Paper no.	II	II	II	II	III	III	III
n	6 **	6 **	13	14	13	8	9
Compartment	BAL	BAL	BAL	BAL	Blood	Blood	Blood
Target	IL-16 mRNA	IL-16 mRNA	IL-16 EC	IL-16 EC	IL-16 EC/IC	IL-16 EC/IC	IL-16 EC/IC
Age (years)	59 (20-70)	63 (55-66)	23 (21-28)	26 (23-44)	66 (39-72)	57 (40-68)	64 (47-68)
Pack-years	0	48 (25-55)	0	***	0	33 (17-46)	36 (21-75)
Male/female	1/5	4/2	6/7	8/6	6/7	4/4	6/3
FEV_1 (% pred.)	98 (86-118)	69 (50-96)	106 (96-129)	106 (95-129)	108 (87-131)	114 (95-129)	58 (30-66)
FEV_1 / FVC	75 (64-96)	62 (42-76)	89 (78-99)	85 (75-99)	78 (65-85)	76 (71-85)	42 (29-56)
DLco (% pred.)	x	x	x	x	96 (80-121)	87 (78-99)	55 (24-71)

**) Some of the subjects are included in the analyses of both rMFI and mRNA.

***) The occasional smokers smoked 1-4 times/month and were only classified in terms of their smoking habits.

EC=extracellular. IC=intracellular. Chr. tob. smokers=chronic tobacco smokers

RESULTS

Increased extracellular IL-16 protein in BAL from long-term tobacco smokers (Paper 1)

There was an increase in soluble extracellular IL-16 protein in BAL samples in both asymptomatic smokers (AS) and those with chronic bronchitis (CB) compared with never-smokers (NS). There was no reproducible difference in extracellular IL-16 protein between AS and CB, or in the CB group with and without airflow obstruction. (Figure 2).

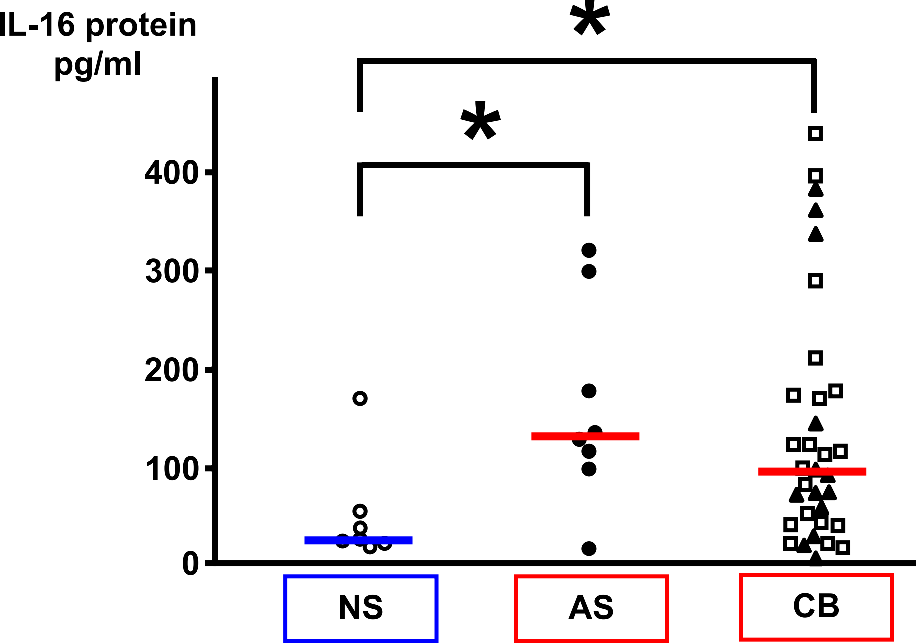


Figure 2. *Concentration of extracellular IL-16 protein in human BAL samples (measured by ELISA) from never-smokers (NS), asymptomatic smokers (AS) and smokers with chronic bronchitis (CB). In the CB group, patients with an* FEV_1 *< 80% pred. are represented by filled triangles and those with an FEV1 >80% pred. by empty squares. Data are presented as median (bold lines) and individual values. * P < 0.05.*

No clear increase in extracellular IL-16 protein in BAL after short-term exposure to tobacco smoke (Paper 2)

Short-term exposure to tobacco smoke did not result in a substantial increase in extracellular IL-16 protein in BAL samples from occasional smokers. BAL 1 was performed before and BAL 2 after short-term smoking. (Figure 3).

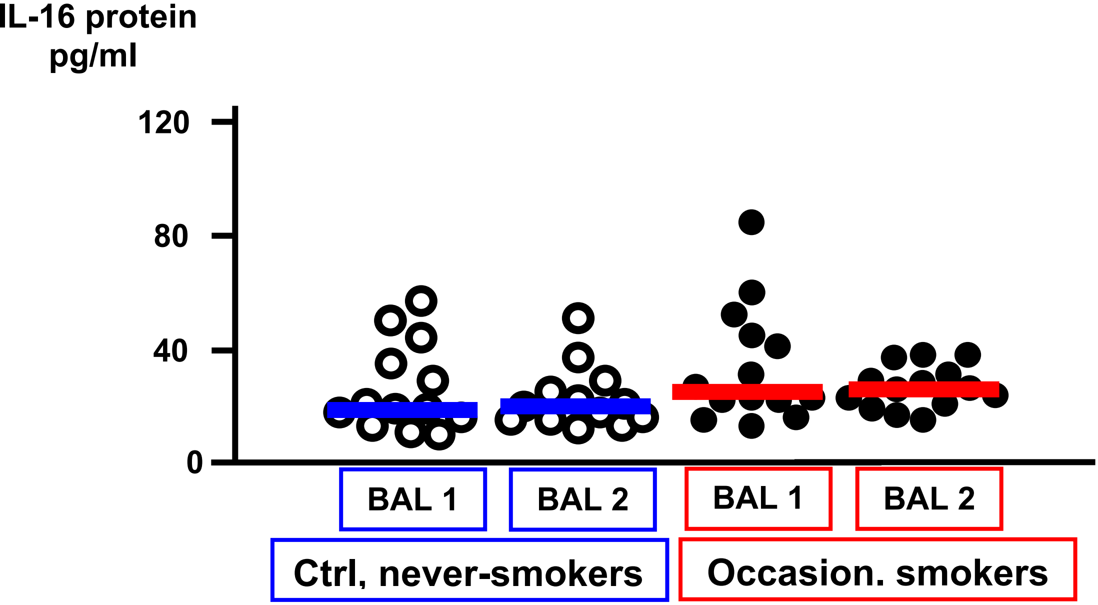


Figure 3. *Concentration of extracellular IL-16 protein in human BAL samples (measured by ELISA) from occasional smokers with and without short-term exposure to tobacco smoke and from controls. Data are presented as median (bold line) and individual values.*

Tobacco smoke decreased IL-16 protein in palatine tonsils (Paper 1)

Tonsil tissue from smokers exhibited a decrease in immunoreactivity (IR) for IL-16 protein compared with that from non-smokers. The assessment of IL-16 IR was performed in tissue samples stained for IL-16 alone and, as a result, we could not decide whether the protein was located in the extra- and/or intracellular space (Figure 4).

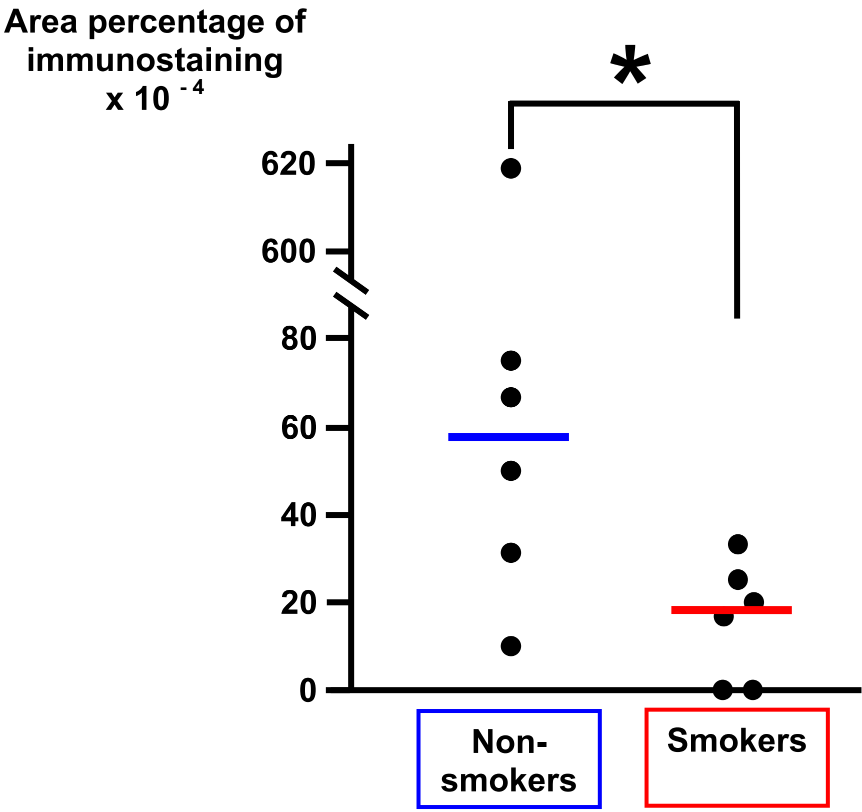


Figure 4. *IR for IL-16 protein in human palatine tonsil tissue from smokers and non-smokers, matched for age. The tissue was treated with membrane permeabilizing agents. The result is expressed as IL-16 IR in % of total area. Data are presented as median (bold lines) and individual values. * P < 0.05.*

CSE caused release of extracellular IL-16 protein from CD8$^+$ cells *in vitro* (Paper 1)

Human blood CD8$^+$ cells stimulated with water-soluble tobacco smoke components (CSE) exhibited an increased concentration of extracellular IL-16 protein in conditioned medium (Figure 5).

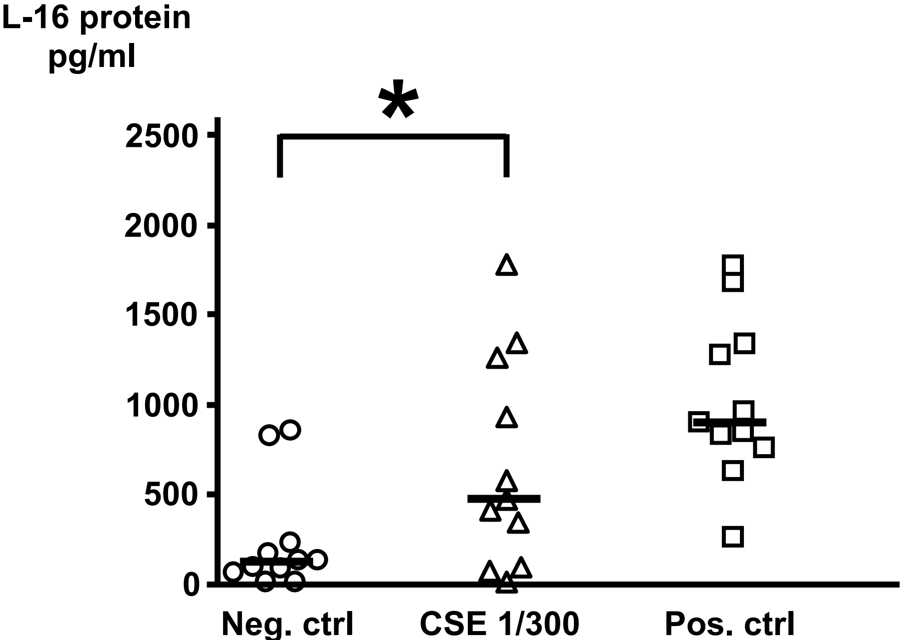


Figure 5. *Concentration of extracellular IL-16 protein in conditioned medium (measured by ELISA), from human CD8$^+$ cells cultured with and without CSE in vitro. Data are presented as median (bold lines) and individual values. * $P < 0.05$.*

Long-term tobacco smoking decreased intracellular IL-16 protein in BAL CD8$^+$ cells (Paper 2)

In chronic tobacco smokers, there was a clear reduction in the intracellular expression of IL-16 protein in BAL CD8$^+$ cells (measured by means of flow cytometry) compared with never-smokers (Figure 6 A-C).

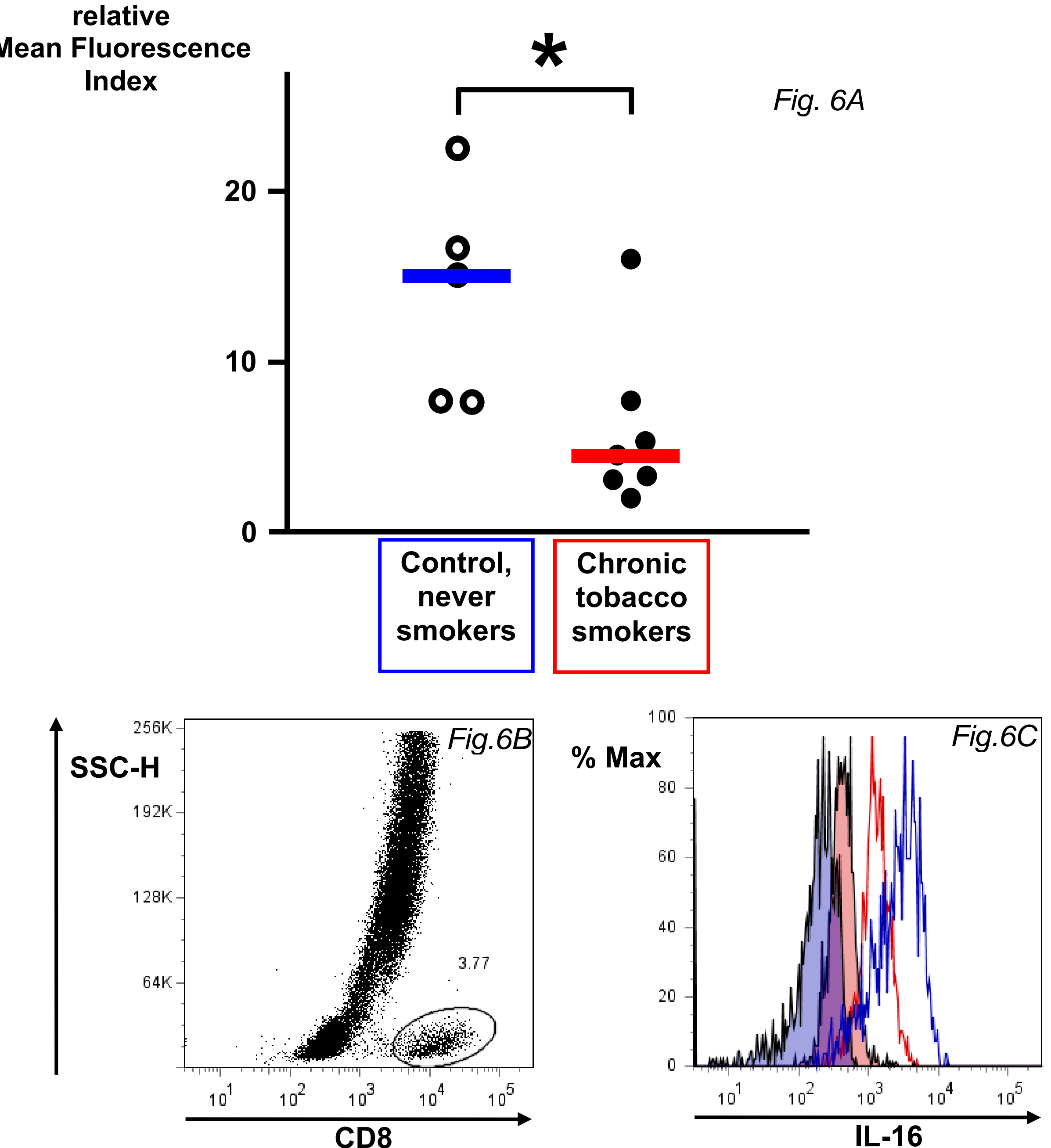


Figure 6A-C. *Flow cytometry analysis of intracellular IL-16 protein in human BAL CD8$^+$ cells* ***A:*** *The concentration of intracellular IL-16 protein is expressed as rMFI (relative Mean Fluorescence Index). Data are presented as median (bold lines) and individual values. * P < 0.05.* ***B:*** *Representative dot plot of BAL cells. Exclusion of 7AAD$^+$ cells was followed by gating of CD45$^+$ and thereafter of CD8$^+$ cells.* ***C:*** *Representative histogram of CD45$^+$CD8$^+$ cells from a smoker (marked red) and a never-smoker (marked blue). The open areas represent the staining for IL-16 protein and the coloured areas indicate the isotype control.*

Long-term tobacco smoking decreased IL-16 mRNA in BAL cells (Paper 2)

There was a slight but reproducible reduction in IL-16 mRNA in BAL cells from long-term tobacco-smokers compared with never-smokers (Figure 7).

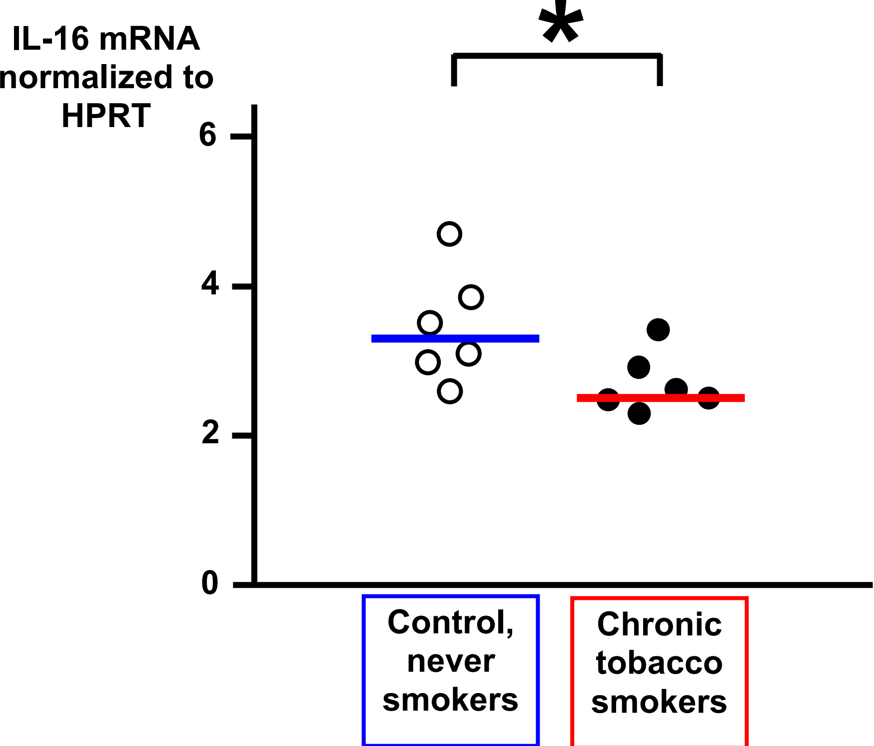


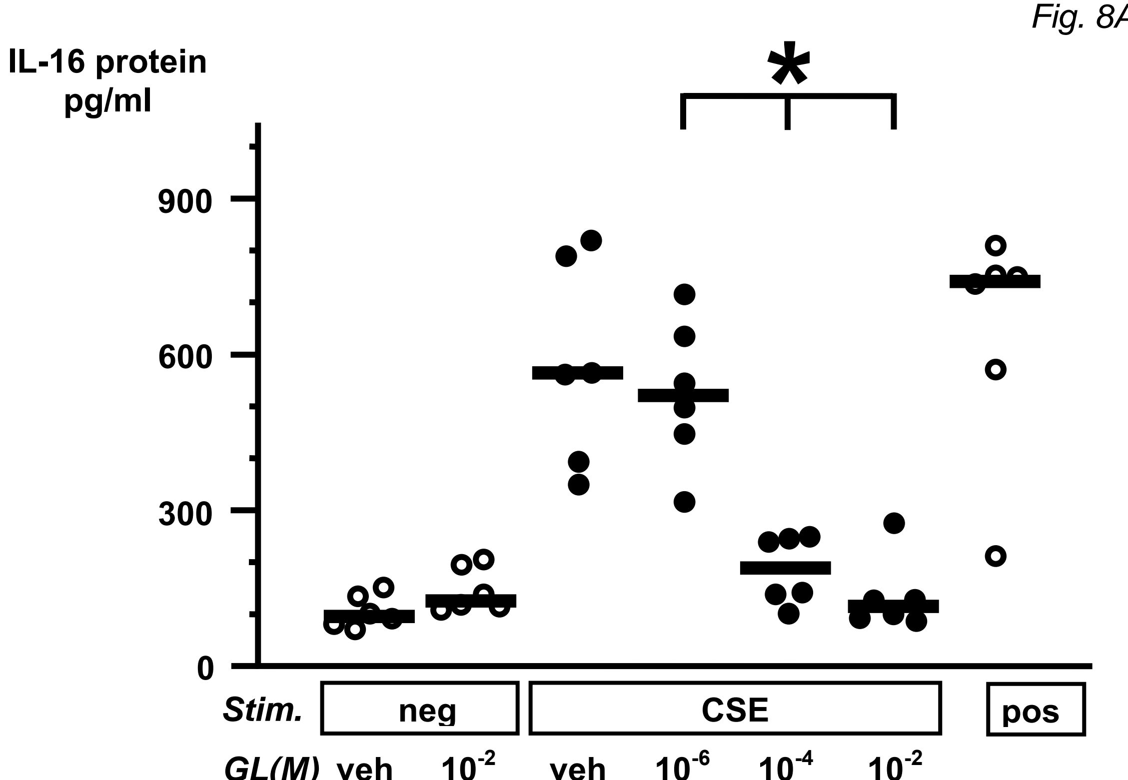
Figure 7. *Levels of IL-16 mRNA in human BAL cells (measured by real-time RT-PCR), from chronic tobacco smokers and controls. Data are presented as median (bold lines) and individual values. * $P < 0.05$.*

Drugs without any clear effect on the CSE-induced increase of extracellular IL-16 protein in $CD8^+$ cells cultured *in vitro* (Paper 2)

The CSE-induced increase in extracellular IL-16 protein in human $CD8^+$ cells in conditioned medium was not affected by a glucocorticoid receptor agonist (hydrocortisone), a β2 agonist (terbutaline) or a combination of both. Nor did a protein synthesis inhibitor (cycloheximide), a calcineurin phosphatase inhibitor (cyclosporin A) or a caspase-3 inhibitor have any effect. See Table 2 and p. L50 in Paper 2.

Glutathione and aminophylline concentration-dependently normalized the CSE-induced increase in extracellular IL-16 protein in $CD8^+$ cells cultured *in vitro* (Paper 2)

When the CSE stimulated $CD8^+$ cells were treated with an oxygen free-radical scavenger (glutathione) or with a nonselective phosphodiesterase inhibitor (aminophylline), there was a concentration-dependent normalisation of the CSE induced release of extracellular IL-16 protein (Figure 8 A-B).



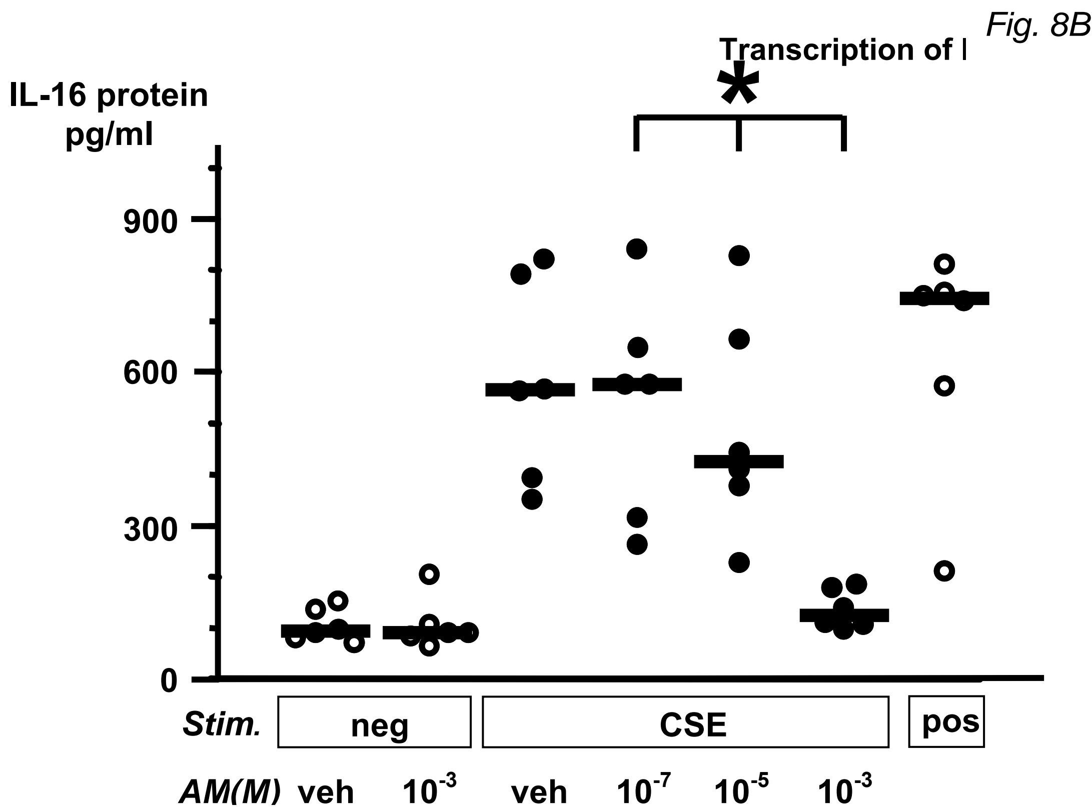


Figure 8 A-B. *Concentrations of extracellular IL-16 protein in conditioned medium (measured by ELISA) from human blood CD8⁺ cells cultured in vitro with CSE (filled circles) and treated with* ***A:*** *glutathione (GL) or* ***B:*** *aminophylline (AM). Data are presented as median (bold lines) and individual values. * P < 0.05.*

Glutathione but not aminophylline normalized the CSE-induced decrease in intracellular IL-16 protein in $CD8^+$ cells *in vitro* (Paper 2)

Exposure to CSE substantially reduced the intracellular concentration of IL-16 protein in $CD8^+$ cells cultured *in vitro* (**Figure 9A**) as well as the number of $IL\text{-}16^+$ $CD8^+$ cells (**Figure 9B**). This was fully reversed by treatment with an oxygen free radical scavenger (glutathione). In contrast, treatment with a nonselective phosphodiesterase (aminophylline) inhibitor did not reverse the process (**Figure 9A-C**).

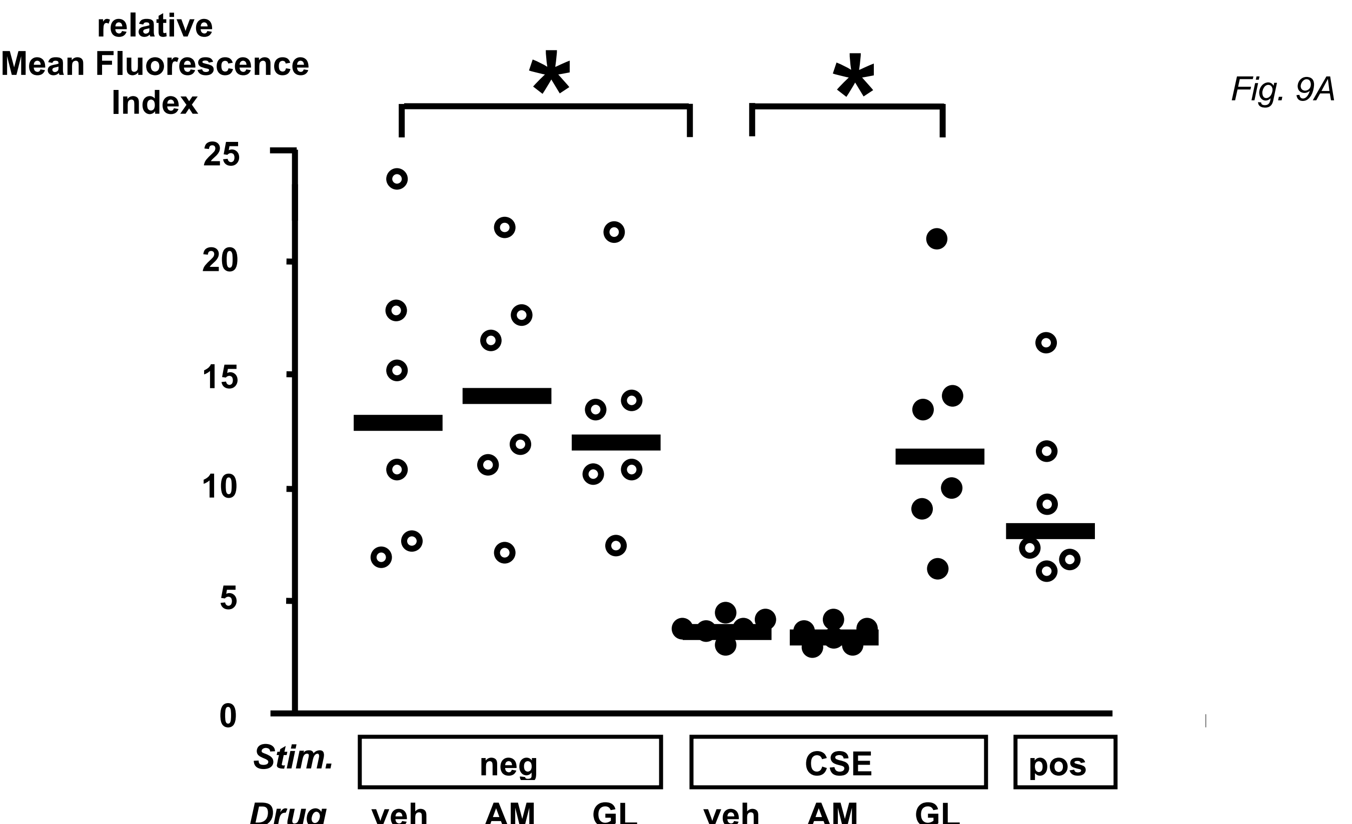


Fig. 9A

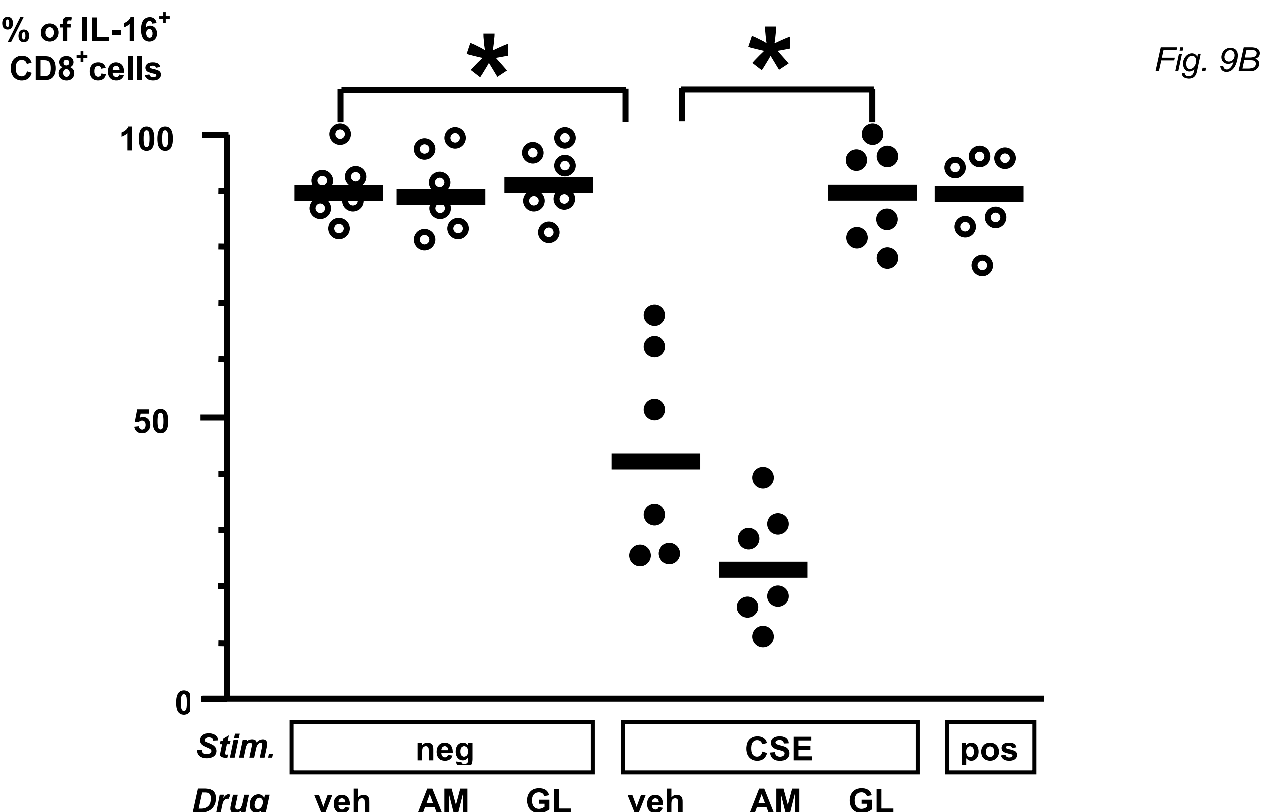
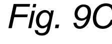


Fig. 9B



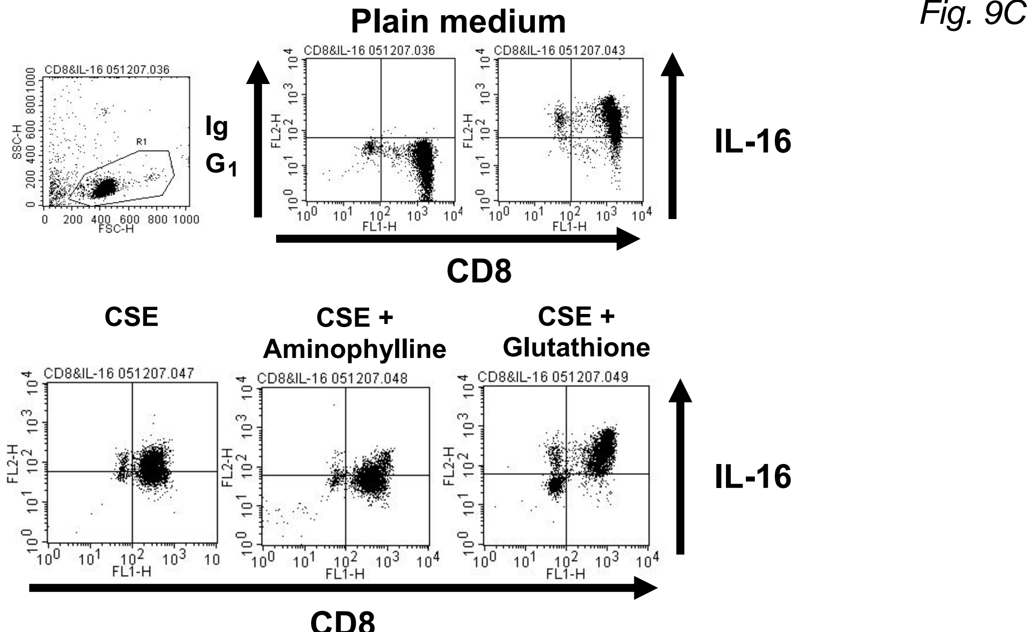


Figure 9A-C. *Flow cytometry analysis of human blood CD8$^+$ cells cultured in vitro with CSE (filled circles) and treated with glutathione (GL) or aminophylline (AM).* ***A:*** *The concentration of intracellular IL-16 protein expressed as rMFI.* ***B:*** *The percentage of CD8$^+$ cells with intracellular IL-16 protein* ***C:*** *The representative flow cytometry recordings. The negative control is composed of IgG$_{1.}$ Data are presented as median (bold lines) and individual values (Figure B-C). * P < 0.05.*

Glutathione normalized while aminophylline aggravated the CSE-induced decrease in intracellular IL-16 mRNA in CD8$^+$ cells *in vitro* (Paper 2)

The decrease in IL-16 mRNA in CSE-stimulated CD8$^+$ cells cultured *in vitro* was totally prevented by treatment with an oxygen free radical scavenger (glutathione). In contrast, treatment with a nonselective phosphodiesterase inhibitor (aminophylline) aggravated the reduction in IL-16 mRNA (Figure 10).

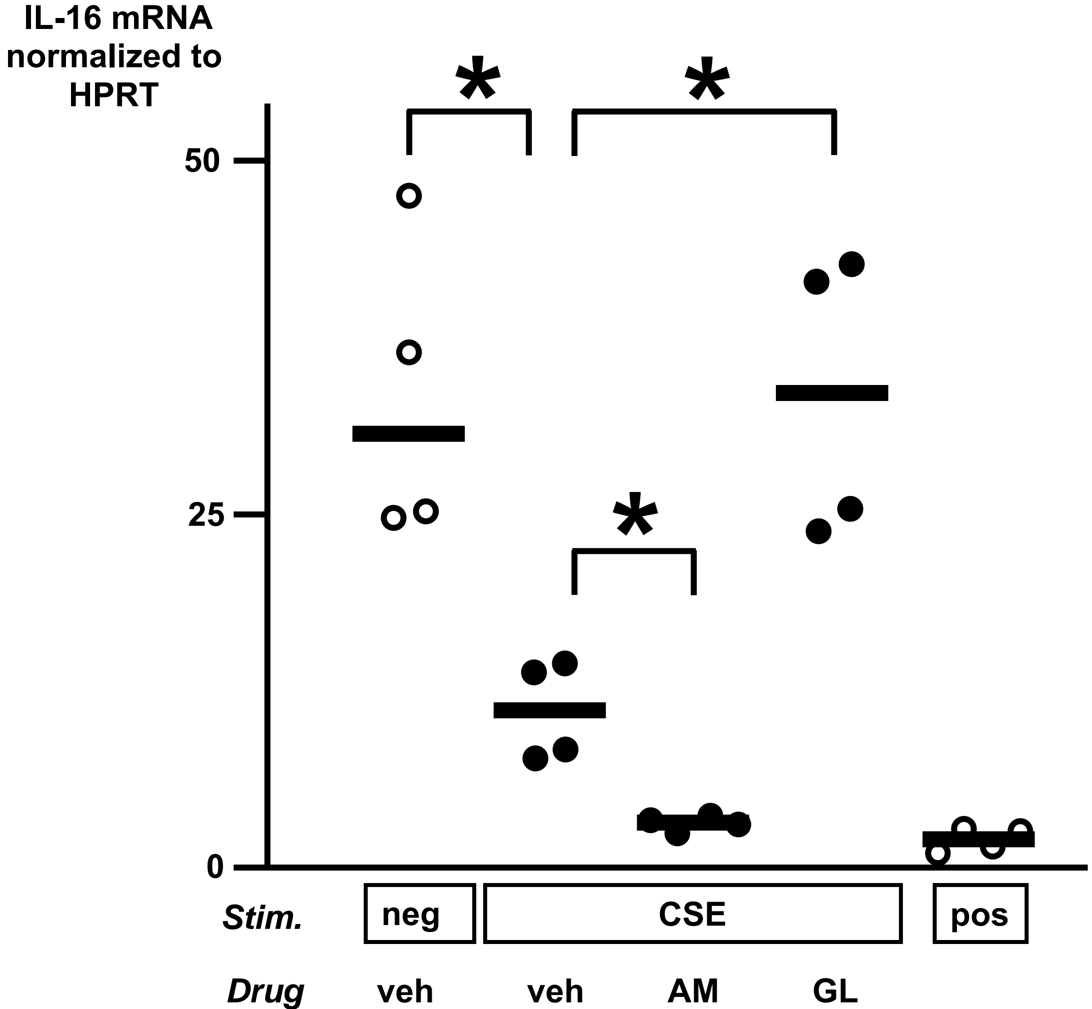


Figure 10. *Levels of IL-16 mRNA in human blood CD8$^+$ cells cultured in vitro (measured by real-time RT-PCR) with CSE (filled circles) and treated with aminophylline (AM) or glutathione (GL). Data are presented as median (bold lines) and individual values. * $P < 0.05$.*

CSE increased oxidized proteins in CD8+ cells *in vitro* (Paper 2)

The amount of oxidized proteins in CSE-stimulated human CD8+ cells *in vitro* increased markedly (Figure 11).

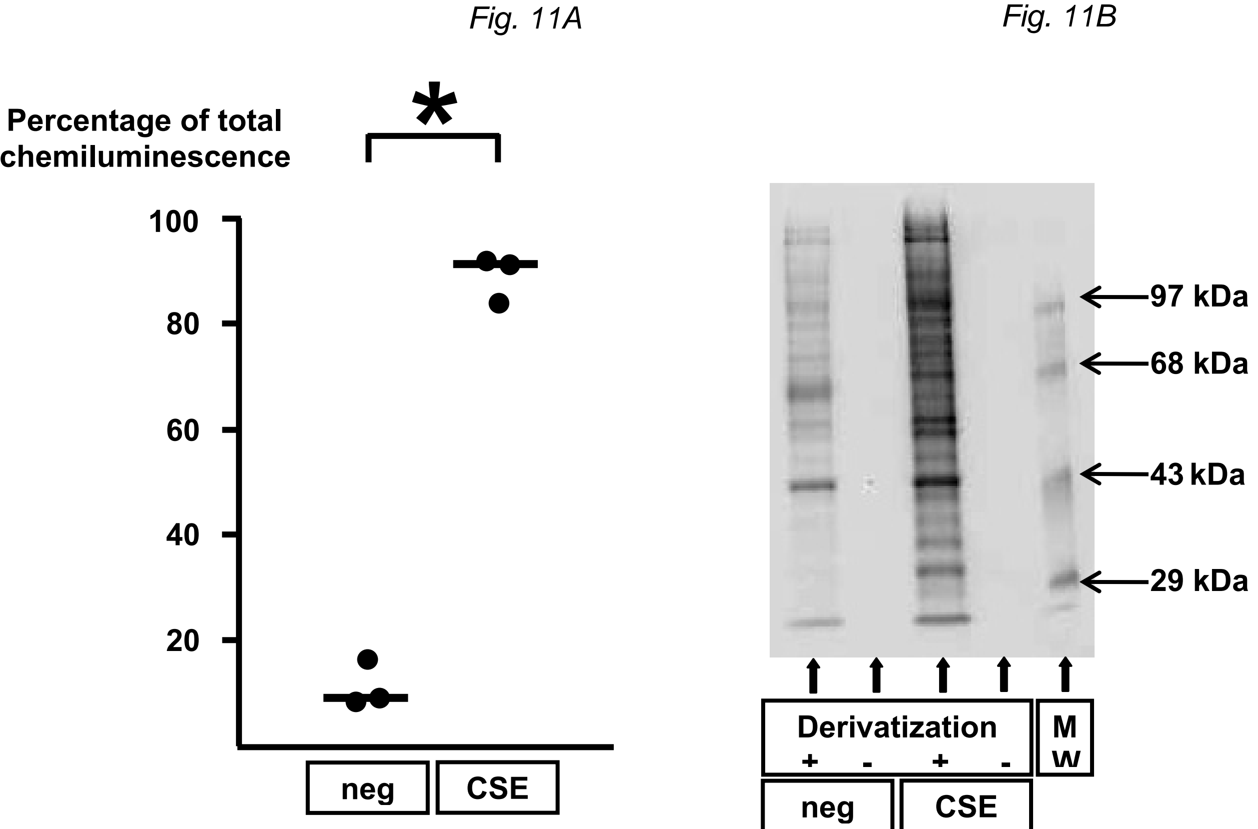
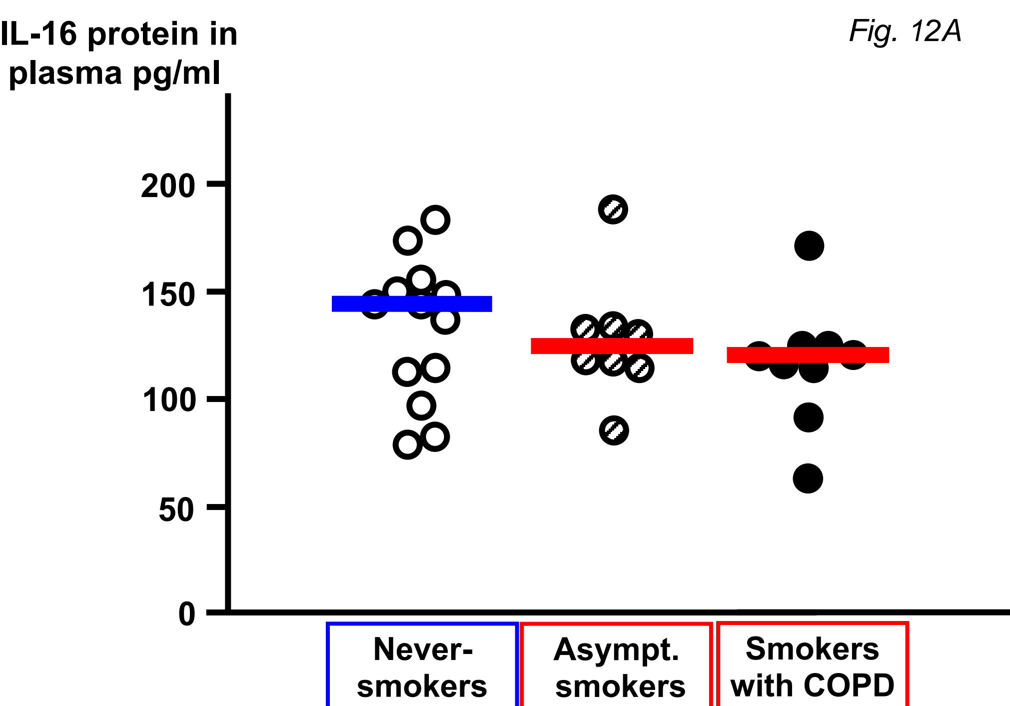


Figure 11 A-B. *The relative amount of oxidized proteins from human blood CD8+ cells cultured in vitro (measured by chemiluminescence), with and without exposure to CSE.* ***A:*** *The relative intensity of the chemiluminescence from the carbonyl groups (%), the presence of which is a result of protein oxidization. Data are presented as median (bold lines) and individual values. * P < 0.05.* ***B:*** *Representative Western blot, with and without DNPH derivatization. A ladder for molecular weight (MW) is illustrated on the right.*

Long-term tobacco smoking did not result in a substantial change in extracellular IL-16 protein in blood (Paper 3)

We detected no substantial alterations in systemic concentrations of extracellular IL-16 protein in plasma or in serum among asymptomatic smokers or smokers with COPD compared with never-smokers (**Figure 12 A-B**).



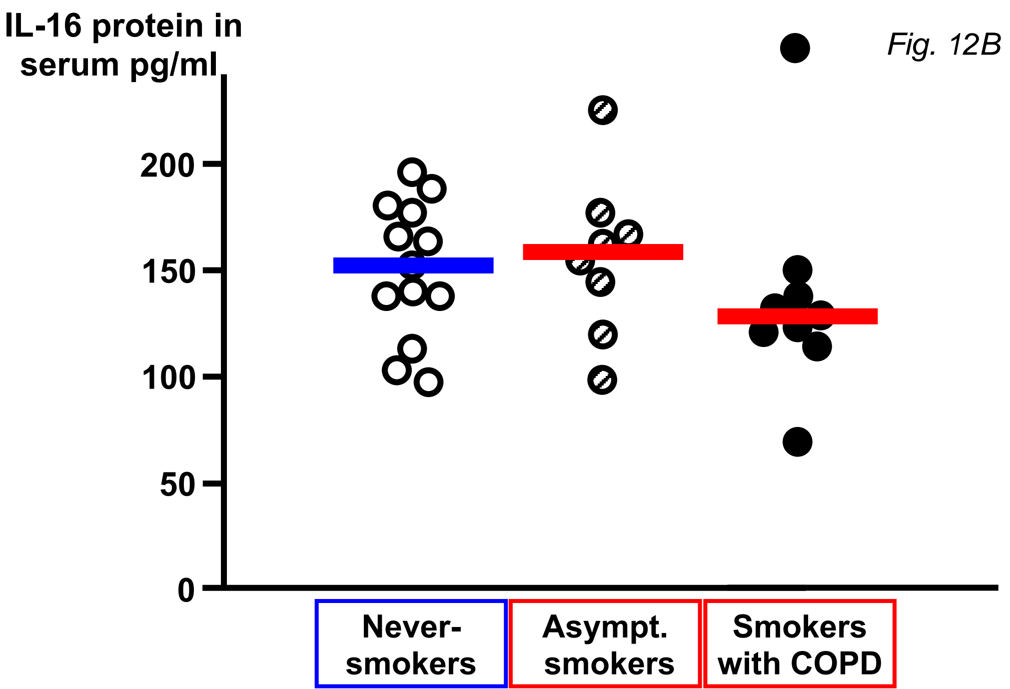


Figure 12 A-B. *IL-16 protein in human blood (measured by ELISA), in plasma and in serum. Data are presented as median (bold lines) and individual values.*

Long-term tobacco smoking did not result in a substantial change in intracellular IL-16 protein in blood CD8$^+$ cells (Paper 3)

There was no clear difference in intracellular IL-16 protein in blood CD8$^+$ cells between never-smokers, asymptomatic smokers or smokers with COPD. See Table 2A in Paper 3).

Summary of results *in vivo*

Analysis/ sample	Extracellular IL-16 protein	Intracellular IL-16 protein in $CD8^+$ cells	Transcription of IL-16 in all BAL cells	IL-16 protein
BAL from long-term tobacco smokers (Paper 1)	↑			
BAL from occasional smokers (Paper 2)	—			
BAL from long-term tobacco smokers (Paper 2)	↑ *)	↓	↓	
Tonsils from tobacco smokers (Paper 1)				↓
Plasma and serum from long-term tobacco smokers (Paper 3)	—	—		

Summary of results in vivo. This summary presents the results from the *in vivo* analysis carried out in 5 different human materials. A green arrow indicates an increase, a red arrow a decrease, while a blue bar indicates no change. *) These results are not presented in this thesis but only in Paper 2.

Summary of results *in vitro*

Analysis/ sample	**Extracellular IL-16 protein**	**Intracellular IL-16 protein**	**Transcription of IL-16**
CSE-treated CD8$^+$ cells *in vitro* (Papers 1 and 2)			
CSE-treated CD8$^+$ cells *in vitro* with glutathione (Paper 2)			
CSE-treated CD8$^+$ cells *in vitro* with aminophylline (Paper 2)			
CSE-treated CD8$^+$ cells *in vitro* with hydrocortisone, terbutaline or both (Paper 2)			
CSE-treated CD8$^+$ cells *in vitro* with cycloheximide, cyclo-sporin A or caspase-3 inhibitor (Paper 2)			

Summary of results in vitro. This summary presents the results from the *in vitro* studies on cultured human CD8$^+$ cells. A green arrow indicates an increase, a red arrow a decrease, while a blue bar indicates no change.

DISCUSSION

The *in vivo* studies presented here demonstrate that tobacco smoke has a considerable impact on IL-16 biology, primarily in the lower airways, and that $CD8^+$ cells are likely to be involved. The corresponding *in vitro* studies elucidate putative mechanisms behind the release of IL-16 protein from $CD8^+$ cells as well as indicating potential targets for preventive treatment.

Local impact of tobacco smoke on IL-16 protein and mRNA in $CD8^+$ cells in the lower airways

Measurement of IL-16 protein in BAL samples from different human materials revealed that current long-term tobacco smokers, with a tobacco load corresponding to at least 10 pack-years, demonstrated an increase in extracellular IL-16 protein in the lower airways [49, 65, 66]. Occasional tobacco smokers did not exhibit such an increase. The exact and thus “critical dose” of tobacco smoke that causes this increase was therefore not identified. However, it may lie between the modest dose represented by “occasional smoking” and the high dose characterised by “long-term tobacco smoking”. Furthermore, our results are compatible with the notion of mainly pre-formed IL-16 protein being released by tobacco smoke components. This is based on two findings from $CD8^+$ cells cultured *in vitro* and treated with water-soluble tobacco smoke components in combination with a protein synthesis inhibitor (cycloheximide) or a caspase-3-inhibitor (caspase-3 cleaves pro-IL-16 into active IL-16) [31, 66]. Neither the protein synthesis inhibitor (cycloheximide) nor the caspase-3-inhibitor affected the release of extracellular IL-16 protein. It seems plausible that when $CD8^+$ cells enter the airways, exposure to locally high doses of tobacco smoke components leads to a transient release of pre-formed IL-16 protein whilst depletion of intracellular IL-16 protein occurs [66] . This is supported by two additional findings. Firstly, the intracellular IL-16

protein concentration is decreased in BAL $CD8^+$ cells from long-term tobacco smokers. Secondly, $CD8^+$ cells stimulated with water-soluble tobacco smoke components *in vitro* exhibit an intracellular decrease in IL-16 protein. Our assumption is also compatible with tobacco smoke components suppressing IL-16 mRNA in $CD8^+$ cells, as shown in our *in vivo* and *in vitro* studies.

Systemic impact of tobacco smoke on extracellular IL-16 protein

When we planned our study to investigate whether long-term tobacco smokers exhibit altered IL-16 protein in plasma or serum, it was uncertain whether the decrease in IL-16 IR observed in palatine tonsils from smokers reflected a local or a systemic impact. This decrease in IL-16 IR among smokers is compatible with tobacco smoke depleting IL-16 protein from the lymphocytes in tonsil tissue. In contrast to the increase in IL-16 protein found in BAL samples from long-term tobacco smokers, we found no substantial difference in extracellular IL-16 protein in plasma or serum from long-term tobacco smokers compared to never-smokers, irrespective of COPD. Taken together, these findings indicate that the effect of long-term tobacco smoking on soluble, extracellular IL-16 protein is more a local than a systemic phenomenon. In line with this, we did not find a difference in intracellular IL-16 protein in $CD8^+$ cells between these groups. However, in our study of systemic effects on IL-16 in long-term tobacco smokers, we found a lower number of blood IL-16^+ NK cells, suggesting that tobacco smoke components can also exert some effects on immune cells at this level.

Cellular source of IL-16 protein in the lower airways

We now know that there are several potential sources of the extracellular IL-16 protein found in the lower airways of long-term tobacco smokers. Many leukocytes, including $CD8^+$ and $CD4^+$ cells, eosinophils, epithelial cells,

monocytes, B cells and NK cells, can express IL-16 protein [28, 67-69]. In a previous study of bronchial biopsy tissue harvested after allergen challenge in patients with asthma, it was demonstrated that IL-16 IR can be detected in T cells and eosinophils [41, 43]. Therefore, when we started the present work, our reasoning was as follows. Since local eosinophilia is mainly linked to atopic airway diseases and not airway inflammation caused by tobacco smoke, T cells constitute a more likely source of IL-16 protein in long-term tobacco smokers than eosinophils. Two findings from our present work support this. Firstly, our *in vitro* studies revealed that water-soluble tobacco smoke components cause the release of IL-16 protein from both $CD8^+$ and $CD4^+$ cells as well as a decrease in IL-16 rMFI and the percentage of $IL\text{-}16^+$ $CD8^+$ cells [65]. Secondly, our *in vivo* study showed that long-term tobacco smokers exhibit a decrease in rMFI $CD8^+$ cells in BAL. The fact that the change in the percentage of $IL\text{-}16^+$ $CD8^+$ cells was not statistically significant *in vivo* may be due to the variability in a relatively limited study population. Another explanation may be that even if tobacco smoke depletes intracellular IL-16 protein in $CD8^+$ cells in the airways, the subgroup of immune cells defined as $IL\text{-}16^+$ $CD8^+$ cells is not affected. It should be emphasized that never-smokers also exhibited measurable concentrations of IL-16 protein in BAL samples. In the *in vitro* experiments, all un-stimulated and cultured $CD8^+$ cells, i.e. negative controls, exhibited a spontaneous release of IL-16 protein. Our measurements confirmed that many blood $CD8^+$ cells contain IL-16 protein and are abundant in BAL samples. Therefore, it seems plausible that these cells are important contributors to the IL-16 protein in the lower airways. In addition, $CD4^+$ cells may also constitute a source of IL-16 protein in long-term tobacco smokers with moderate to severe COPD. We believe that this is the case for two reasons: First, it has been reported that this group of COPD patients has an increased number of $CD4^+$ cells in the airways [14-16]. Second, in our present work, we demonstrated that water-soluble tobacco smoke components lead to the release of IL-16 protein from $CD4^+$ cells *in vitro* [65].

In yet another earlier study by Laberge *et al.* it was claimed that epithelial cells in the bronchial mucosa exhibit IL-16 IR in patients with asthma [41]. When we tried to reproduce this finding in bronchial biopsies from long-term tobacco smokers, we were unable to detect any IL-16 IR; despite utilising the very same protocol that successfully detected IL-16 IR tissue in palatine tonsils (data not published).

Plausible mechanisms behind the release of IL-16 protein

Interestingly, both glutathione and aminophylline attenuated the induced release of extracellular IL-16 protein caused by the water-soluble tobacco smoke components (CSE) in a concentration dependent manner. However, aminophylline, which is a non-selective phosphodiesterase inhibitor, did not affect the CSE-induced decrease in the intracellular concentration of IL-16 measured as rMFI. Moreover, compared with the control group of $CD8^+$ cells exposed to CSE without any drug treatment, treatment with aminophylline decreased the number of $IL\text{-}16^+CD8^+$ cells as well as the IL-16 mRNA in these cells. Glutathione, which is an oxygen free radical scavenger, prevented both of the above-mentioned effects of CSE. There may be at least two explanations for the attenuated release of extracellular IL-16 protein caused by aminophylline in CSE-treated $CD8^+$ cells: First, aminophylline may prevent the release of intracellular protein into the extracellular space. Second, our findings clearly indicate a suppressive effect on IL-16 mRNA and therefore the observed decrease in intracellular IL-16 protein may be partly a consequence of inhibited production of IL-16 protein. In other words, taken together, it is possible that the observed attenuation of CSE-induced release of extracellular IL-16 protein after treatment with aminophylline is due to less intracellular IL-16 protein being available for release into the extracellular space. Notably, we also detected a high level of protein oxidation in $CD8^+$ cells *in vitro* caused by CSE. Overall, these results support the assumption that the effect of tobacco smoke on IL-16 in $CD8^+$ cells is dependent on the generation of oxygen free

radicals. The clear protective effect of glutathione treatment on CSE-exposed cells *in vitro* further supports this.

Biological implications of the local increase in IL-16 protein in the lower airways

The only previous study on tobacco smokers by Laan *et al.* demonstrated a negative correlation between the concentration of IL-16 protein in BAL and the percentage of $CD4^+$ cells in blood [49]. It was speculated that this finding is compatible with $CD4^+$ cells migrating into the lung towards the IL-16 gradient. The increase in $CD4^+$ cells found in the airways of tobacco smokers, at least in those with COPD, may also depend upon a local proliferation of $CD4^+$ cells [14-16]. The increase in IL-16 protein in the airways of long-term tobacco smokers may contribute to a local proliferation of $CD4^+$ cells as indicated by an *in vitro* study [36]. As mentioned in the Introduction, there are several studies suggesting that IL-16 is a pro-inflammatory cytokine, while other studies mainly regard it as an anti-inflammatory cytokine [37, 38]. Two recent studies have shown that suppressive T regulatory cells (i.e. $CD4^+CD25^+$ cells) may dominate in the airways of smokers and are more susceptible to the chemo-attractant IL-16 [70, 71]. If future studies can establish that these suppressive cells accumulate in the airways of smokers, it may be helpful for controlling tobacco-smoked induced airway inflammation.

IL-16 and lung function

In the group of CB patients, we did not find any difference in extracellular IL-16 protein between those with FEV_1 >80 % pred. and FEV_1<80% pred. In other human material examined in our study, there was no correlation between lung function (FEV_1 % pred.) and the concentration of extracellular IL-16 protein [66]. Taken together, our findings are compatible with the assumption that the dividing line between those who exhibited an increased IL-16 concentration in

the lower airways and those who did not is smoking or not smoking, irrespective of COPD.

Modulating IL-16 in lung diseases

Interleukin-16 has not yet been used for treatment of human airway disease. However, some conclusions on its therapeutic potential can be drawn from animal studies. Treatment with IL-16 antibodies in mouse models of encephalomyelitis, renal ischemia-reperfusion injury and ischemic limb injury blocks the influx of $CD4^+$ cells [39, 40, 72]. This inhibition caused by IL-16 improved the outcome in the first two studies in terms of reversed paralysis and reduced concentrations of serum creatinine in blood, respectively. In the study of ischemic limb injury, the outcome was worse when collateral development was impaired. In this case, the influx of $CD4^+$ cells was essential. Furthermore, a peptide that blocked the CD4 binding part of IL-16 was administered intranasally in a mouse model of airway hyperresponsiveness, resulting in an improvement [73]. Additional studies are required before we know whether blocking or increasing IL-16 in the airways is a way forward for patients with airway inflammation caused by tobacco smoke. One critical question is whether or not the influx of $CD4^+$ cells in the airways is desirable. In asthma, there is a correlation between the severity of disease and the number of $CD4^+$ cells in the airways [74]. Since previous studies have shown an increase in both IL-16 and $CD4^+$ cells in the airways of asthmatics, blocking IL-16 may be one strategy, at least for those who are difficult to treat [41, 42]. However, we know far less about the role of the local increase in $CD4^+$ cells in the airways of long-term tobacco smokers with moderate to severe COPD than we do about patients with asthma. Clearly, new interventional studies targeting IL-16 are required, starting with animal models of airway inflammation caused by tobacco smoke.

SUMMARY AND CONCLUSIONS

With regard to our initial hypothesis, our present work on humans and human cells confirms that tobacco smoke affects the production and release of IL-16 protein in $CD8^+$ cells and that the impact on IL-16 is most pronounced locally in the airways.

1. We confirmed that long-term tobacco smokers exhibited an increase in extracellular IL-16 protein in the airways. Short-term tobacco smokers did not have this increase. Tobacco smokers also exhibited a decrease in IL-16 protein in lymphoid tissue.

2. The water-soluble tobacco smoke components (CSE) can release extracellular IL-16 protein from $CD8^+$ cells. Thus, these cells constitute a source of IL-16 protein.

3. Tobacco smoke and water-soluble tobacco smoke components deplete intracellular IL-16 protein in $CD8^+$ cells both *in vitro* and *in vivo* as well as reducing IL-16 mRNA transcription in $CD8^+$ cells *in vitro* and in BAL cells *in vivo*.

4. Water-soluble components of tobacco smoke cause the release of extracellular IL-16 protein, which can be prevented by an oxygen free radical scavenger (OFR; glutathione) and a non-selective phosphodiesterase inhibitor (aminophylline), respectively. Thus, this prevention is likely to involve both OFRs and some phosphodiesterases. The water-soluble components of tobacco smoke that cause decreased transcription of IL-16 mRNA as well as intracellular IL-16 protein were only prevented by OFRs. It is therefore likely that OFRs and not phosphodiesterases are involved in this process, despite

the fact that the latter may play a role in the release of extracellular IL-16 protein.

5. Long-term tobacco smokers with and without COPD do not exhibit a substantial alteration in extracellular IL-16 protein in plasma or serum, which suggests a mainly local effect of tobacco smoke in terms of IL-16

TACK!

Anders Lindén, min sympatiske huvudhandledare, vill jag först och främst tacka för denna tioåriga forskningsresa som vi har gjort tillsammans. Alltid tillhands för att diskutera forskningsspörsmål med även om Du har varit bortrest, haft semester eller varit strängt upptagen av den kliniska verksamheten. Tack även för våra intressanta samtal om livets allehanda ting. Och tack för Din outtröttliga förmåga att försöka förbättra min engelska!

Varmt tack till min bihandledare **Margareta Sjöstrand** som lärde mig allt från cellodlingar till andra laborationstekniker. En klok, allmänbildad, omtänksam och intressant person som jag har haft glädjen att bli vän med. En renässansmänniska som turligt nog lever i detta århundrade!

Jag hade lyckan att begåvas med ännu en bihandledare då jag måtte ha varit ett särdeles svårt fall. Stort tack **Ingemar Qvarfordt** som alltid har stöttat mig och givit kloka synpunkter!

Ett särskilt tack vill jag rikta till **Carina Malmhäll** för Dina kunskaper i olika slags cellfärgningar och mRNA-analyser och till **Apostolos Bossios** för Dina kunskaper i flödescytometri, som ni så båda generöst har delgivit mig. Och tack för de trevliga personer som ni är att ha att göra med!

Tack **Bengt-Eric Skoogh**, dåvarande professor på avdelningen som trodde på mig som forskare och antog mig.

Ett stort tack för era insatser till mina artiklar **Birgitta Houltz** och **Maria Eldh!** Det har verkligen varit en glädje att arbeta med er. Hoppas vi kan göra det igen! Sedan vill jag omnämna de andra medarbetarna till mina artiklar, **Martti Laan, Gerdt Riise, Pernilla Glader, Britt-Marie Eldh, Lars-Olof Cardell** och **Bengt Andersson**.

Och icke att förglömma medlemmarna i den forna forskningsgruppen LFG samt nuvarande LIM. **Jan Lötvall, Madeleine Rådinger, Anna-Karin Dahlborn** (som lärde mig kongresskunskap i Atlanta, vilket innebar hur man hittade både föreläsningssal, gratis lunch och bra pennor), **Cecilia Lässer, You Lu, Madeleine Ahrnens, Elin Silverpil, Marit Hansson** och **Pernilla Glader**.

Jag är även ett tack skyldig till sköterskorna på lungdiagnostiska avdelningen som var behjälpliga med både information om lämpliga studiepatienter och med provtagning till mitt andra manuskript. Tack **Jeanette Sundberg**, **Eva Erriahi, Kristina Johansson, Monika Crona** och **Mia Johansson**. Tack även till mina kollegor som hjälpte till med bronkoskopiprovtagningarna. Stort och varmt tack till **Dorota Persson, Lory Fägerström** och **Eric Fortes Lähdet** för era fina insatser till mitt tredje manuskript.

Merci madame **Kristina Andelid!** Tillsammans har vi de senaste åren slitit och våndats med våra vetenskapliga alster under de mest konstiga tider på elevhemmet, dock med avbrott för en och annan fikapaus. Tack för Din vänskap och för Din aldrig sinande ström av geléhallon!

I en klinisk vardag med allestädes närvarande läkarbrist på schemaraden vill jag tacka verksamhetschefen **Mona Palmqvist** och schemaläggarna **Margareta Smith** och **Anita Nordenson** att ni trots detta har sett till att jag har fått forskningstid. Tack till alla mina kollegor på Lungkliniken och Allergologen som gör detta till en sådan trevlig, vänlig och stimulerande plats att arbeta på!

Jag är även tacksam till dem som ställde upp som studiepatienter som därigenom gjorde denna avhandling möjlig!

Stort, stort, stort tack till mina underbara föräldrar **Bernt** och **Barbro Andersson** som alltid har stöttat mig och trott på min forskning även i perioder av kräftgång! Och till min kära syster **Karin** och svåger **Martin Ahlström** med familj, min älskade faster **Barbro Carlsson** och min älskade mormor **Berta Ericsson**.

Mina nära och kära vänner **Krister Myhrman**, **Ingela Hammar** och **Jakob Lindblad**. Och **Anita Ternberg** samt **Bengt Gustafsson**! Mina gamla biologkompisar **Martin Larsvik** (jag lovar att bli en flitigare skrivare av Frideborgsbrev nu när jag har disputerat!) och **Erland Lettevall**. Inte att förglömma ”gänget” med **Marie Glassell**, **Jennifer** och **Staffan Sandgren**, **Gisela** och **Niklas Höjer**, **Per Gyllén** och **Göran Jungersten**.

I detta sammanhang vill jag även rikta ett tack till Lasarettet i Skene där jag fick en sådan bra start på min läkarbana och där en bit av mitt hjärta alltjämt dröjer sig kvar. Tack **Rakel Tengel** för Din klokhet som läkare, mentor och medmänniska och för Din vänskap. Och tack **Lotte Waller** för den goda handledningen som Du gav mig där och till **Britt-Marie Eldh** för god handledning på lungkliniken!

Ett stort tack till övrig släkt och vänner som ej är omnämnda här!

Och sist men inte minst, stort tack till min livskamrat **Eric Fortes Lähdet!** Under alla dessa år som jag har hållit på med min forskning har Du aldrig knotat en enda gång när jag har behövt jobba länge på konstiga tider eller då jag har skördat prover från mina cellodlingar mitt i natten.

REFERENCES

1. *Pelkonen, M., Smoking: relationship to chronic bronchitis, chronic obstructive pulmonary disease and mortality. Curr Opin Pulm Med, 2008.* ***14****(2): p. 105-9.*
2. *GLOBAL STRATEGY FOR THE DIAGNOSIS, MANAGEMENT, AND PREVENTION OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE. www.goldcopd.com, 2010.*
3. *Raherison, C. and P.O. Girodet, Epidemiology of COPD. Eur Respir Rev, 2009.* ***18****(114): p. 213-21.*
4. *Raherison, C., Epidémiologie de la bronchopneumopathie chronique obstructive. Rev Prat.* ***61****(6): p. 769-73.*
5. *Marquis, K., F. Maltais, and P. Poirier, Les manifestations cardiovasculaires chez les patients atteints d´une BPCO. Rev Mal Respir, 2008.* ***25****(6): p. 663-73.*
6. *Lehouck, A., et al., BPCO et métabolisme osseux: une mise à jour clinique Rev Mal Respir.* ***27****(10): p. 1231-42.*
7. *Pauly, J.L. and G. Paszkiewicz, Cigarette smoke, bacteria, mold, microbial toxins, and chronic lung inflammation. J Oncol.* ***2011****: p. 819129.*
8. *Mortaz, E., G. Folkerts, and F. Redegeld, Mast cells and COPD. Pulm Pharmacol Ther.* ***24****(4): p. 367-72.*
9. *Geng, Y., et al., Effects of nicotine on the immune response. II. Chronic nicotine treatment induces T cell anergy. J Immunol, 1996.* ***156****(7): p. 2384-90.*
10. *Li, Q., et al., Inhibition of human T lymphoblast proliferation by hydroquinone. Toxicol Appl Pharmacol, 1996.* ***139****(2): p. 317-23.*
11. *Shah, K.A. and H.T. Karnes, A review of the analysis of tobacco-specific nitrosamines in biological matrices. Crit Rev Toxicol.* ***40****(4): p. 305-27.*
12. *Dekhuijzen, P.N., Antioxidant properties of N-acetylcysteine: their relevance in relation to chronic obstructive pulmonary disease. Eur Respir J, 2004.* ***23****(4): p. 629-36.*
13. *O'Donnell, R., et al., Inflammatory cells in the airways in COPD. Thorax, 2006.* ***61****(5): p. 448-54.*
14. *Hogg, J.C., et al., The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med, 2004.* ***350****(26): p. 2645-53.*
15. *Turato, G., et al., Airway inflammation in severe chronic obstructive pulmonary disease: relationship with lung function and radiologic emphysema. Am J Respir Crit Care Med, 2002.* ***166****(1): p. 105-10.*
16. *Lofdahl, M.J., et al., Increased intraepithelial T-cells in stable COPD. Respir Med, 2008.* ***102****(12): p. 1812-8.*

17. *Nonaka, M. and H. Satake, Urochordate immunity. Adv Exp Med Biol.* ***708****: p. 302-10.*
18. *Parker, D. and A. Prince, Innate immunity in the respiratory epithelium. Am J Respir Cell Mol Biol.* ***45****(2): p. 189-201.*
19. *Rabson, A., I. Roitt, and J. Delves, MEDICAL IMMUNOLOGY. Second edition, Blackwell Publishing, 2005.*
20. *Guerin, J.C., et al., Le stress oxydatif en pathologie bronchopulmonaire: apport de la N-acétyl-cystéine (NAC). Rev Pneumol Clin, 2005.* ***61****(1 Pt 1): p. 16-21.*
21. *Ivanov, S. and A. Linden, Interleukin-17 as a drug target in human disease. Trends Pharmacol Sci, 2009.* ***30****(2): p. 95-103.*
22. *Krensky, A.M. and C. Clayberger, Biology and clinical relevance of granulysin. Tissue Antigens, 2009.* ***73****(3): p. 193-8.*
23. *Finkelstein, R., et al., Alveolar inflammation and its relation to emphysema in smokers. Am J Respir Crit Care Med, 1995.* ***152****(5 Pt 1): p. 1666-72.*
24. *Saetta, M., et al., CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med, 1999.* ***160****(2): p. 711-7.*
25. *Ekberg-Jansson, A., et al., The expression of lymphocyte surface antigens in bronchial biopsies, bronchoalveolar lavage cells and blood cells in healthy smoking and never-smoking men, 60 years old. Respir Med, 2000.* ***94****(3): p. 264-72.*
26. *Center, D.M. and W. Cruikshank, Modulation of lymphocyte migration by human lymphokines. I. Identification and characterization of chemoattractant activity for lymphocytes from mitogen-stimulated mononuclear cells. J Immunol, 1982.* ***128****(6): p. 2563-8.*
27. *Cruikshank, W. and D.M. Center, Modulation of lymphocyte migration by human lymphokines. II. Purification of a lymphotactic factor (LCF). J Immunol, 1982.* ***128****(6): p. 2569-74.*
28. *Cruikshank, W.W., H. Kornfeld, and D.M. Center, Interleukin-16. J Leukoc Biol, 2000.* ***67****(6): p. 757-66.*
29. *Berman, J.S., et al., Chemoattractant lymphokines specific for the helper/inducer T-lymphocyte subset. Cell Immunol, 1985.* ***95****(1): p. 105-12.*
30. *Wu, D.M., et al., Processing and release of IL-16 from CD4+ but not CD8+ T cells is activation dependent. J Immunol, 1999.* ***162****(3): p. 1287-93.*
31. *Zhang, Y., et al., Processing and activation of pro-interleukin-16 by caspase-3. J Biol Chem, 1998.* ***273****(2): p. 1144-9.*
32. *Laberge, S., et al., Secretion of IL-16 (lymphocyte chemoattractant factor) from serotonin-stimulated CD8+ T cells in vitro. J Immunol, 1996.* ***156****(1): p. 310-5.*
33. *Laberge, S., et al., Histamine-induced secretion of lymphocyte chemoattractant factor from CD8+ T cells is independent of transcription and translation. Evidence for constitutive protein synthesis and storage. J Immunol, 1995.* ***155****(6): p. 2902-10.*

34. *Liu, C., et al., IL-16 signaling specifically induces STAT6 activation through CD4. Cytokine, 2007.* ***38****(3): p. 145-50.*
35. *Qin, X.J., et al., Interleukin-16 in tuberculous and malignant pleural effusions. Eur Respir J, 2005.* ***25****(4): p. 605-11.*
36. *Parada, N.A., et al., Synergistic activation of CD4+ T cells by IL-16 and IL-2. J Immunol, 1998.* ***160****(5): p. 2115-20.*
37. *Mathy, N.L., et al., Interleukin-16 stimulates the expression and production of pro-inflammatory cytokines by human monocytes. Immunology, 2000.* ***100****(1): p. 63-9.*
38. *Klimiuk, P.A., J.J. Goronzy, and C.M. Weyand, IL-16 as an anti-inflammatory cytokine in rheumatoid synovitis. J Immunol, 1999.* ***162****(7): p. 4293-9.*
39. *Skundric, D.S., et al., Anti-IL-16 therapy reduces CD4+ T-cell infiltration and improves paralysis and histopathology of relapsing EAE. J Neurosci Res, 2005.* ***79****(5): p. 680-93.*
40. *Wang, S., et al., Decreased renal ischemia-reperfusion injury by IL-16 inactivation. Kidney Int, 2008.* ***73****(3): p. 318-26.*
41. *Laberge, S., et al., Increased expression of interleukin-16 in bronchial mucosa of subjects with atopic asthma. Am J Respir Cell Mol Biol, 1997.* ***17****(2): p. 193-202.*
42. *Cruikshank, W.W., et al., Early identification of interleukin-16 (lymphocyte chemoattractant factor) and macrophage inflammatory protein 1 alpha (MIP1 alpha) in bronchoalveolar lavage fluid of antigen-challenged asthmatics. Am J Respir Cell Mol Biol, 1995.* ***13****(6): p. 738-47.*
43. *Laberge, S., et al., Increased expression of IL-16 immunoreactivity in bronchial mucosa after segmental allergen challenge in patients with asthma. J Allergy Clin Immunol, 2000.* ***106****(2): p. 293-301.*
44. *Laan, M., A. Linden, and G.C. Riise, IL-16 in the airways of lung allograft recipients with acute rejection or obliterative bronchiolitis. Clin Exp Immunol, 2003.* ***133****(2): p. 290-6.*
45. *Alexandrakis, M.G., et al., Serum level of interleukin-16 in multiple myeloma patients and its relationship to disease activity. Am J Hematol, 2004.* ***75****(2): p. 101-6.*
46. *Blaschke, S., et al., Interleukin 16 expression in relation to disease activity in rheumatoid arthritis. J Rheumatol, 2001.* ***28****(1): p. 12-21.*
47. *Lee, S., et al., Circulating interleukin-16 in systemic lupus erythematosus. Br J Rheumatol, 1998.* ***37****(12): p. 1334-7.*
48. *Shimonkevitz, R., et al., Interleukin-16 expression in the peripheral blood and CD8 T lymphocytes after traumatic injury. J Trauma, 2005.* ***58****(2): p. 252-8.*
49. *Laan, M., et al., Increased levels of interleukin-16 in the airways of tobacco smokers: relationship with peripheral blood T lymphocytes. Thorax, 1999.* ***54****(10): p. 911-6.*
50. *Lu, N.Z., et al., International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily:*

glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev, 2006. ***58****(4): p. 782-97.*

51. *Barnes, P.J., Glucocorticosteroids: current and future directions. Br J Pharmacol, 2011.* ***163****(1): p. 29-43.*
52. *Läkemedelsverket, Farmakologisk behandling av kroniskt obstruktiv lungsjukdom - KOL. Ny rekommendation. Information från Läkemedelsverket 2:2009, 2008.*
53. *Barnes, P.J., Distribution of receptor targets in the lung. Proc Am Thorac Soc, 2004.* ***1****(4): p. 345-51.*
54. *Loza, M.J. and R.B. Penn, Regulation of T cells in airway disease by beta-agonist. Front Biosci (Schol Ed), 2010.* ***2****: p. 969-79.*
55. *Chen, C.Y., et al., Effect of oral aminophylline on pulmonary function improvement and tolerability in different age groups of COPD patients. Chest, 2005.* ***128****(4): p. 2088-92.*
56. *Myou, S., et al., Bronchodilator effects of intravenous olprinone, a phosphodiesterase 3 inhibitor, with and without aminophylline in asthmatic patients. Br J Clin Pharmacol, 2003.* ***55****(4): p. 341-6.*
57. *Barnes, P.J., Theophylline in chronic obstructive pulmonary disease: new horizons. Proc Am Thorac Soc, 2005.* ***2****(4): p. 334-9; discussion 340-1.*
58. *Cantin, A.M., et al., Normal alveolar epithelial lining fluid contains high levels of glutathione. J Appl Physiol, 1987.* ***63****(1): p. 152-7.*
59. *Decramer, M., et al., Effects of N-acetylcysteine on outcomes in chronic obstructive pulmonary disease (Bronchitis Randomized on NAC Cost-Utility Study, BRONCUS): a randomised placebo-controlled trial. Lancet, 2005.* ***365****(9470): p. 1552-60.*
60. *Arima, M., et al., Expression of interleukin-16 by human epithelial cells. Inhibition by dexamethasone. Am J Respir Cell Mol Biol, 1999.* ***21****(6): p. 684-92.*
61. *Murphy, P., T. Hillman, and K. Rajakulasingam, Therapeutic targets for persistent airway inflammation in refractory asthma. Biomed Pharmacother.* ***64****(2): p. 140-5.*
62. *Amor, K.T., C. Ryan, and A. Menter, The use of cyclosporine in dermatology: part I. J Am Acad Dermatol, 2010.* ***63****(6): p. 925-46; quiz 947-8.*
63. *Budihardjo, I., et al., Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol, 1999.* ***15****: p. 269-90.*
64. *Ludwiczek, O., et al., Activation of caspase-3 by interferon alpha causes interleukin-16 secretion but fails to modulate activation induced cell death. Eur Cytokine Netw, 2001.* ***12****(3): p. 478-86.*
65. *Andersson, A., et al., Impact of tobacco smoke on interleukin-16 protein in human airways, lymphoid tissue and T lymphocytes. Clin Exp Immunol, 2004.* ***138****(1): p. 75-82.*
66. *Andersson, A., et al., Effects of tobacco smoke on IL-16 in CD8+ cells from human airways and blood: a key role for oxygen free radicals? Am J Physiol Lung Cell Mol Physiol.* ***300****(1): p. L43-55.*

67. *Elssner, A., et al., IL-16 is constitutively present in peripheral blood monocytes and spontaneously released during apoptosis. J Immunol, 2004. **172**(12): p. 7721-5.*

68. *Kaser, A., et al., B lymphocyte-derived IL-16 attracts dendritic cells and Th cells. J Immunol, 2000. **165**(5): p. 2474-80.*

69. *Meagher, C., et al., Neutralization of interleukin-16 protects nonobese diabetic mice from autoimmune type 1 diabetes by a CCL4-dependent mechanism. Diabetes, 2010. **59**(11): p. 2862-71.*

70. *McFadden, C., et al., Preferential migration of T regulatory cells induced by IL-16. J Immunol, 2007. **179**(10): p. 6439-45.*

71. *Smyth, L.J., et al., CD4-regulatory cells in COPD patients. Chest, 2007. **132**(1): p. 156-63.*

72. *Stabile, E., et al., CD8+ T lymphocytes regulate the arteriogenic response to ischemia by infiltrating the site of collateral vessel development and recruiting CD4+ mononuclear cells through the expression of interleukin-16. Circulation, 2006. **113**(1): p. 118-24.*

73. *de Bie, J.J., et al., Effect of interleukin-16-blocking peptide on parameters of allergic asthma in a murine model. Eur J Pharmacol, 1999. **383**(2): p. 189-96.*

74. *Nakagome, K. and M. Nagata, Pathogenesis of airway inflammation in bronchial asthma. Auris Nasus Larynx. **38**(5): p. 555-63.*