Effects of tobacco smoke on IL-16 in CD8+ cells from human airways and blood: a key role for oxygen free radicals?

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1Lung Immunology Group, Department of Internal Medicine/Respiratory Medicine and Allergology, 2Krefting Research Centre, Department of Internal Medicine, Institute of Medicine, and 3Department of Microbiology and Immunology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

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Andersson A, Bossios A, Malmhäll C, Sjöstrand M, Eldh M, Eldh B, Glader P, Andersson B, Qvarfordt I, Rüse GC, Lindén A. 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: L43–L55, 2011. First published October 29, 2010; doi:10.1152/ajplung.00387.2009.—Chronic exposure to tobacco smoke leads to an increase in the frequency of infections and in the number of CD8+ and CD4+ cells as well as the CD4+ chemotractant cytokine IL-16 in the airways. Here, we investigated whether tobacco smoke depletes intracellular IL-16 protein and inhibits de novo production of IL-16 in CD8+ cells from human airways and blood while increasing extracellular IL-16 and whether oxygen free radicals (OFR) are involved. Intracellular IL-16 protein in CD8+ cells and mRNA in all cells was decreased in bronchoalveolar lavage (BAL) samples from chronic smokers. This was also the case in blood from chronic smokers and in human blood CD8+ cells exposed to water-soluble tobacco smoke components in vitro. This was not observed in occasional smokers after short-term exposure to tobacco smoke. A marker of activation (CD69) was slightly increased, whereas other markers of cellular functions (membrane integrity, apoptosis, and proliferation) in human blood CD8+ cells in vitro were negatively affected by water-soluble tobacco smoke components. An OFR scavenger prevented these effects, whereas a protein synthesis inhibitor, a β-adrenoceptor, a glucocorticoid receptor agonist, a phosphodiesterase, a calcineurin phosphatase, and a caspase-3 inhibitor did not. In conclusion, we have also shown that intracellular IL-16 protein is decreased in CD8+ cells exposed to water-soluble tobacco smoke components in vitro. However, despite this extracellular increase, we have also shown that intracellular IL-16 protein is decreased in palatine tonsils from chronic tobacco smokers (1). Based on these findings, we hypothesized that tobacco smoke depletes intracellular IL-16 protein and inhibits de novo production of IL-16 in CD8+ cells from human airways and blood while increasing extracellular IL-16 and that oxygen free radicals (OFR) are involved in mediating these effects. The main aim of the current study was to evaluate this specific hypothesis and whether the events caused by tobacco smoke can be prevented by pharmacological means.

For the current study, we harvested bronchoalveolar lavage (BAL) samples from chronic smokers and from never-smokers as well as from occasional smokers with and without prior short-term exposure to tobacco smoke. In chronic smokers and never-smokers, we assessed intracellular IL-16 protein and mRNA, in CD8+ and in all BAL cells, respectively. We also determined the concentrations of extracellular IL-16 and IL-1β protein in the BAL samples from occasional smokers. To identify cellular mechanisms involved in controlling these IL-16 parameters, we exposed human blood CD8+ cells to water-soluble components of tobacco smoke in vitro, with and without various pharmacological interventions. These interventions included treatment with an OFR scavenger, more specifically in those with severe chronic obstructive pulmonary disease (COPD) (16, 38).
ger, a protein synthesis inhibitor, a β-adrenoceptor, a glucocor-
ticoid receptor agonist, a phosphodiesterase, a calcineurin
phosphatase, and a caspase-3 inhibitor, respectively. We also
assessed protein oxidation caused by water-soluble compo-
ments of tobacco smoke in our in vitro system. Finally, we
characterized the impact of these tobacco smoke components
on the key cellular functions (i.e., cell activation, membrane
integrity, apoptosis, and proliferation) of the human CD8+ cells in vitro with and without the same pharmacological
interventions.

METHODS

Samples from Chronic Smokers

Study population. The study was approved by the Regional Ethics
Committee (REC; no. S-093-02). The subjects received oral and
written information and gave their oral and written consent. The
inclusion criterion for chronic smokers was current smokers with a
tobacco exposure corresponding to ≥ 10 pack years. The control group
consisted of never-smokers. Bronchoscopy was performed on a clin-
ical basis due to chronic cough, hemoptysis, or an antibiotic-resistant lung infiltrate. Apart from that, no subject was allowed to have any
clinical sign of infection during the previous 4 wk. The subjects underwent a physician’s evaluation of history and physical findings as
well as the results from laboratory tests before the bronchoscopy. The
clinical characteristics of the subjects are presented in Table 1, Groups
1–3. The harvested material of each individual subject was not
sufficient to allow all cellular analyses, and, because of this, each
individual subject contributed to one, two, or several cellular analyses,
depending on the amount of BAL material obtained.

BAL samples. Bronchoscopy was performed in accordance with
standard clinical procedures previously described (29). Briefly, BAL was
conducted by instillation of 100 ml of isotonic NaCl. The BAL
samples were harvested from the contralateral lung in cases of a focal
X-ray infiltrate. BAL recovery was recorded, and the BAL solution
(fluid and cells) was filtered through a nylon web (70-μm pore size)
for retention of mucus. The BAL sample was then centrifuged (300 g,
10 min, 4°C), and the BAL cell pellet was subsequently used for
measurement of intracellular IL-16 protein in CD8+ cells and for
IL-16 mRNA in BAL cells (see below). The cell-free supernatant was
separated and frozen (−80°C) until measurement of IL-16, IL-1β,
IL-4, IFN-γ, and TNF protein in this BAL fluid.

Intracellular IL-16 protein in CD8+ cells. Immediately after har-
vest, 2 × 10⁶ BAL cells from the cell pellet were dissolved in 0.25 ml
of PBS followed by 200 μl of 0.5% human IgG (Sigma-Aldrich, St.
Louis, MO) and 2.5 μl of a protein transport inhibitor (GolgiStop; BD
Biosciences, Bedford, MA) and incubated (4°C, 15 min) to optimize
the detection of intracellular proteins. Flow cytometry analysis was
performed as previously described, with some modifications as fol-

<table>
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<th>Group</th>
<th>Clinical characteristics</th>
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<td>FEV₁/FVC</td>
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Table 1. Clinical characteristics

Results are presented as medians with ranges. Group 1: 5 never-smokers and 7 chronic smokers who were analyzed for intracellular IL-16 protein (relative mean fluorescence index, rMFI) in bronchoalveolar lavage (BAL) CD8+ cells. Group 2: 6 never-smokers and 6 chronic smokers analyzed for IL-16 mRNA in BAL cells. Group 3: 11 never-smokers and 12 chronic smokers analyzed for extracellular IL-16, IL-1β, TNF, IFN-γ, and IFN-γ protein in BAL samples. Group 4: 13 never-smokers and 14 occasional smokers with and without short-term exposure to tobacco smoke who underwent repeated bronchoscopy and BAL for the detection of extracellular IL-16 protein in BAL samples. FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

IL-16 mRNA. An RNA-stabilization solution (RNAlater; Ambion,
Austin, TX) was added to 2 × 10⁶ BAL cells. The cells were then kept
frozen (−80°C) until further processing. Total RNA purification was
performed with an RNeasy Mini Kit (Qiagen, Hilden, Germany)
followed by DNase treatment using a TURBO DNA-free Kit (Ambi-
ion). The quantity and purity of the eluted total RNA was measured
by means of spectrophotometry (260/280 nm; SpectraMax Plus;
Molecular Devices, Sunnyvale, CA). Each sample (20 μl) of cDNA
was prepared from the mixture of total RNA (100 ng), random
primer (dN)6 (2 μl), Protector RNase Inhibitor (20 units), PCR
Nucleotide MixPLUS (2 μl), and Transcriptor Reverse Transcriptase
(10 units), respectively (all from Roche, Basel, Switzerland). A
GeneAmp PCR System 2400 (PerkinElmer, Wellesley, MA) was used
for synthesis (10 min at 25°C; 30 min at 55°C; 5 min at 85°C). Gene
sequences for human (h) IL-16 and the HPRT reference gene were
accessed from the National Center for Biotechnology Information

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Downloaded from http://ajplung.physiology.org/ by 10.220.36.13 on November 6, 2017
NCBI) database as previously described (1). The gene primer sequences used were as follows:

HPRT: forward, 5'-ATC AGA CTG AAG AGC TAT TGT AAT GAC CA-3'; reverse, 5'-TGG CTT ATA TCC AAC ACT TCG TG-3';

IL-16: forward, 5'-ACC TCA ACT CCT CCA CTG ACT CT-3'; reverse, 5'-TGA TGA TGT TCC AGG CTT CAA AC-3'.

Real-time RT-PCR was performed in duplicates on a LightCycler 2.0 instrument (Roche) using 2-μl cDNA, 500 nM sequence-specific primers (TIB MOLBIOL Syntheslabor, Berlin, Germany), and 4 μl of LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche). The process was carried out by denaturation (95°C, 10 s), annealing (65°C, 5 s), and extension (72°C, 10 s) and was finalized by a dissociation step. In parallel with the testing of the samples, we ran controls for PCR (interrun calibrator), samples without RT, as well as samples without template control. IL-16 mRNA expression was normalized to HPRT and related to the interrun calibrator expression of IL-16 and HPRT using RelQuant Software (Roche).

**Extracellular IL-16, IL-1β, IL-4, IFN-γ, and TNF protein.** The cell-free BAL supernatant (BAL fluid) was handled as previously described (1). Briefly, it was concentrated using ultrafiltration (≥10-fold; Amicon Ultra-4, Ultracel-10k; Millipore, Billerica, MA). The concentration of IL-16 protein was measured using an ELISA kit (BioSource, Camarillo, CO) (1). In all samples, the concentration of IL-16 protein exceeded the lowest standard curve concentration (i.e.,

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**Fig. 1.** Flow cytometry analysis of human CD8+ cells harvested through bronchoalveolar lavage (BAL) in vivo. A: the relative mean fluorescence index (rMFI) for intracellular IL-16 protein. Results for intracellular IL-16 are presented as individual and median values (bold lines). *P < 0.05 (Mann-Whitney U test). B: a representative dot plot of BAL cells. Intact BAL cells were gated by exclusion of 7-amino-actinomycin D (7-AAD)-positive cells, followed by gating of CD45+ and subsequently CD8+ cells (3.77% of CD45+ as marked in the eclipse). C: intracellular IL-16 expression (rMFI) was estimated in CD8+ cells and was correlated with expression of the isotype control IgG2a. A representative histogram of CD45+CD8+ cells from a smoker (red) and a never-smoker (blue) stained for either IL-16 (open area) or IgG2a (tinted area). Smokers displayed a smaller increase in IL-16 expression from isotype control compared with the never-smokers. SSC-H, side scatter height; PE, phycoerythrin; Max, maximum.
>23.4 pg/ml). Moreover, the concentrations of IL-1β, IL-4, IFN-γ, and TNF protein were analyzed in a FACSARia Flow Cytometer, using a CBA Flex Set system (BD Biosciences). For this method, the detection limit was 0.5 pg/ml for IL-1β, 1 pg/ml for IL-4, 0.25 pg/ml for IFN-γ, and 0.25 pg/ml for TNF. IL-1β was detected in 22 out of 23 samples, whereas TNF was detected in only 2 out of 23 samples (data not shown). IL-4 and IFN-γ could not be detected at all. After the measurement of protein concentrations, the results were corrected for the sample concentration procedure. The presented data thus show concentrations in original, nonconcentrated samples.

Samples from Occasional Smokers

Study population. This part of the study was also approved by the REC (no. T441-02). The subjects received oral and written information and gave their oral and written consent. The full clinical protocol as well as other data sets than those presented in the current study have been published elsewhere (13). In essence, occasional smokers with normal lung function (limited smoking exposure 1–4 times/month) and not within 4 wk before the study) and never-smokers underwent 2 bronchoscopies including BAL. BAL samples were harvested on days 1 and 14. On days 12 and 13, the included subjects smoked either 10 cigarettes (occasional smokers) or none (never-smokers). The cigarettes were of a commercial brand (Marlboro; Philip Morris, Neuchâtel, Switzerland), purchased commercially, and not provided as a gift. The standardized dose of cigarette smoke was chosen as ethically imperative. For this part of the study, we prepared and analyzed the cell-free BAL supernatant (BAL fluid) with respect to the concentration of extracellular IL-16 protein only (see above). The clinical characteristics of the included subjects are presented in Table 1, Group 4.

Samples from Blood CD8+ Cells Cultured In Vitro

Enrichment of CD8+ cells. Human buffy coat preparations were obtained from healthy blood donors as previously described (1). The blood samples were diluted in PBS (1:3), and the peripheral blood mononuclear cells were collected through density centrifugation over a Ficoll gradient (Pharmacia Biotech, Uppsala, Sweden). Subsequently, the CD8+ cells were enriched using a negative magnetic separation technique (CDS+ T Cell Isolation Kit II; Milleniy Biotec, Bergisch Gladbach, Germany). Cytospin slides were prepared, air-dried, and stored frozen until immunochemistry was performed for purity control.

Preparation of water-soluble components of tobacco smoke. We generated water-soluble components of tobacco smoke in the form of cigarette smoke extract (CSE) as described previously (1, 35). Briefly, we prepared CSE by drawing the mainstream smoke from 10 medium nicotine and tar commercial cigarettes (Marlboro) through 15 ml of RPMI 1640 (Sigma-Aldrich), thereby extracting the water-soluble components (1, 35). Each cigarette was “vacuum-smoked” for 3 min. The CSE solution was thereafter passed through a sterile filter, divided into aliquots, and stored frozen (−80°C) until the experiments were conducted. The chosen CSE concentration (a 1:10 dilution) yielded reproducible IL-16 responses with no detrimental effect on cell viability (see RESULTS). Notably, the endotoxin concentration in CSE was <1 pg/ml when analyzed by a colorimetric kinetic method (Endochrome-K; Charles River Endosafe, Charleston, SC).

Purity and viability of enriched CD8+ cells. After enrichment, the purity of the CD8+ cells before culture was analyzed by means of immunochemistry staining, as previously described (1). The median purity (median with range) of CD8+ cells was 91% (88–94%, n = 3). The corresponding viability of CD8+ cells was 100% (100–100%, n = 3) as judged by the exclusion of trypan blue (1). The viability was also assessed at the time of the harvesting of conditioned medium after 20 h in the experiments on extracellular IL-16 protein (below). With reference to CD8+ cell viability, there was no substantial reduction of this parameter caused by the concentration causing maximum impact for either treatment (median percentage with range, n = 6): 100 (99–100) for CSE + 10−6 M glutathione vs. 100 (98–100) for CSE alone; 100 (99–100) for CSE + 10−3 M aminophylline vs. 100 (98–100) for CSE alone; 100 (95–100) for CSE + 10−4 M cycloheximide vs. 100 (98–100) for CSE alone and 100 (99–100) for CSE + 10−7 M cyclosporine A vs. 100 (98–100) for CSE alone.

Moreover, the corresponding viability data (median percentage with range, n = 4–6) for additional treatments were: 100 (98–100) for CSE + 10−8 M hydrocortisone vs. 100 (99–100) for CSE alone and 100 (95–100) for CSE + 10−4 M terbutaline vs. 100 (99–100) for CSE alone. Finally, for the experiments with the capsaicin-3 inhibitor, the corresponding viability data (median percentage with range, n = 5–7) were: 97 (94–98) for CSE + 10−7 M capsaicin-3 inhibitor vs. 96 (94–98) for CSE.

Culture of CD8+ cells. The enriched CD8+ cells were cultured in complete medium, as previously described, and were exposed to CSE (a 1:10 dilution in the complete medium) alone or together with the respective treatment (1). The complete medium served as a negative control, alone or together with the highest used concentration of the respective drug. Stimulation with calcium ionophore (C2; 1 μM) and PMA (2 ng/ml; Sigma-Aldrich) was used as a technical control. Depending on the experiment, the CD8+ cells were cultured for 2.5, 5, 10, 20, or 72 h in a humidified incubator (5% CO2, 37°C).

Pharmacological interventions. Drugs (Sigma-Aldrich) and CSE were added to the CD8+ cells. The vehicle solution (negative control) corresponded to that required for the highest used drug concentration. The CD8+ cells were cotreated with one of the following: an OFR scavenger ([l-glutathione; 10−6, 10−4, and 10−2 M] (18, 40); a nonselective phosphodiesterase inhibitor (aminophylline; 10−7, 10−5, and 10−3 M) (17); a protein synthesis inhibitor (cycloheximide; 10−8, 10−6, and 10−4 M) (3); a calcineurin phosphatase inhibitor (cyclosporine A; 10−8, 10−7, and 10−6 M) (2); a glucocorticoid receptor agonist (hydrocortisone; 10−8, 10−6, and 10−4 M) (3); or a β-adrenergic receptor agonist (terbutaline; 10−8, 10−6, and 10−4 M) (36). Ethanol was used as a solvent for both cycloheximide and cyclosporine. The final vehicle concentration of ethanol did not exceed 0.1%, and this concentration was used as the control vehicle in the negative and positive controls.

Fig. 2. Levels of human IL-16 mRNA in BAL cells harvested in vivo, normalized to the reference gene (HPRT) and measured by real-time RT-PCR. The results are presented as individual and median values (bold lines). *P < 0.05 (Mann-Whitney U test).
positive control groups. For the cotreatment experiments, hydrocortisone and terbutaline were added simultaneously (10^{-6} M for both drugs). Because of the fact that, of the tested drugs, only glutathione and aminophylline exerted effects on the concentrations of extracellular IL-16, these drugs were employed (10^{-2} and 10^{-3} M, respectively) in the subsequent experiments on intracellular IL-16 protein, IL-16 mRNA, activation, membrane integrity, apoptosis, and proliferation. In separate in vitro experiments, a caspase-3 inhibitor (10^{-6}, 10^{-5}, and 10^{-4} M; Z-DEVD-FMK; R&D Systems, Minneapolis, MN) was used to evaluate impact on CSE-induced release of extracellular IL-16 protein (42). In these experiments, the drug was originally dissolved in DMSO, and the highest concentration of DMSO was therefore included in the corresponding negative (vehicle only) and positive (CSE) control treatments.

Protein oxidation in vitro. The degree of protein oxidation was determined in the CD8^{+} cells cultured for 20 h by Western blot analysis to demonstrate the involvement of OFR, and thus oxidative stress, in the response to CSE (9). Briefly, total proteins were extracted from the CD8^{+} cells (n = 3) by using modified RIPA buffer (15) and sonication. Cell debris was then removed by centrifugation (300 g, 5 min, 4°C). Detection and quantification of the relative amount of oxidized proteins was performed using a commercial kit (OxyBlot Oxidized Protein Detection Kit; Millipore) according to the manufacturer’s recommendations. This method detects the protein carbonyl groups for which presence constitutes a consequence of the molecular modification caused by OFR (9). In all experiments, standardized amounts (10 μg) of total protein were derivatized with 2,4-dinitrophenylhydrazine (DNPH) and directly loaded onto a polyacrylamide gel and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Finally, the membranes were blotted using primary and secondary Abs specific to the OxyBlot kit. Negative controls (no DNPH) were run in parallel with each sample. Detection and relative quantification were performed using enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden) and Quantity One software (Bio-Rad). For each experiment, the total intensity was set to 100% for the sum of the intensity of the negative control and the CSE treatment together.

Intracellular IL-16 protein in vitro. The CD8^{+} cells were cultured for 20 h, and GolgiStop was added 6 h before harvest. The cell culture, cell staining, as well as the flow cytometry analysis for intracellular IL-16 protein and calculation of rMFI were performed as described above. Anti-CD8 Ab (clone SK1) conjugated with peridinin-chloro-

Fig. 3. Concentrations of extracellular IL-16 (A) and IL-1β (B) protein measured with ELISA in BAL samples from chronic smokers (*P < 0.05, Mann-Whitney U test). C: scattergram for IL-16 protein and IL-1β protein in BAL samples (Spearman rank correlation, *P < 0.05, rho = 0.54). D: concentrations of extracellular IL-16 protein in BAL samples from occasional smokers with and without short-term exposure to tobacco smoke (*P > 0.05, Mann-Whitney U test). Never-smokers are indicated by means of ○ and chronic smokers by ●. The results are presented as individual as well as median values (bold lines).
phyll-protein complex (PerCP) or FITC with their respective isotype control and anti-IL-16 Ab (clone 14.1) conjugated with PE were used for the experiment. Assessments of activation, membrane integrity, and apoptosis were also performed simultaneously (see below).

IL-16 mRNA in vitro. The analysis of IL-16 mRNA in CD8\(^+\) cells was performed after culture for 10 h in duplicate wells under the conditions described above. The harvested CD8\(^+\) cells (1 \times 10^6) were centrifuged twice (1,000 g, 5 min), RNA later was added, and the samples were kept frozen (−80°C) before the actual analysis was performed (same procedure as above).

Extracellular IL-16 protein in vitro. The conditioned medium was carefully collected after culture for 2.5, 5, 10, or 20 h. At the time of harvest, the remaining cells were removed through centrifugation (300 g, 10 min, 20°C), and the cell-free supernatant was frozen (−80°C). In uncentrifuged samples, the concentration of IL-16 protein was assessed (same procedure as above).

Cell activation in vitro. To determine cell activity, the surface expression of anti-CD69 Ab (clone L78)-PerCP (BD Biosciences) was analyzed in the CD8\(^+\) cells after 20 h of culture (same procedure as above).

Cell membrane integrity in vitro. The intracellular marker 7-AAD was used after being cultured for 20 h to assess the membrane damage in the CD8\(^+\) cells (same procedure as above).

Apoptosis in vitro. To determine the apoptosis, the CD8\(^+\) cells were stained after 20 h of culture with the intracellular apoptosis marker anti-active caspase-3 Ab (clone C92-605) FITC (BD Biosciences; same procedure as above).

Cell proliferation in vitro. The enriched CD8\(^+\) cells were cultured for 72 h in triplicate wells, as described above. A solution containing radioactive [6-\(^3\)H]thymidine (10 \mu l diluted 1:10 in RPMI; Amersham, Buckinghamshire, United Kingdom) was added 18 h before harvest. At the end of the experiment, the cells were frozen (−80°C) in the culture plates. After thawing, the cells were collected on a filter (Printed Filtermat A; PerkinElmer) using a Cell Harvester (FilterMate D961962; PerkinElmer). Following drying, the filter was sealed by a Cell Harvester (FilterMate D961962; PerkinElmer) using a Cell Harvester (FilterMate D961962; PerkinElmer). Radioactivity was assessed (1 min) and expressed as counts per minute.

Statistical Analysis of Data

The data were analyzed by using standard computer statistics software (StatView 4.01; Abacus Concepts, Berkeley, CA). The Kruskal-Wallis test was used for comparison between multiple groups, followed by the Mann-Whitney U test. Correlation analysis was performed with the Spearman rank correlation test. Wilcoxon signed rank test was used for the evaluation of the experiments on time course and caspase-3 inhibition. The paired t-test was used for the analysis of data on protein oxidation. A P value of <0.05 was regarded as statistically significant. Throughout the manuscript, n = number of independent experiments (equating the number of subjects or blood donators investigated).

RESULTS

Samples from Chronic Smokers

Chronic smokers exhibited a reduction in intracellular IL-16 protein (expressed as rMFI) compared with never-smokers (Fig. 1, A–C), whereas the percentage of IL-16\(^+\)CD8\(^+\) cells in BAL samples did not markedly differ for these 2 groups (data not shown). Chronic smokers also exhibited lower IL-16 mRNA than never-smokers (Fig. 2). The concentrations of extracellular IL-16 and IL-1β protein were higher in BAL samples from chronic smokers than in the corresponding samples from never-smokers (Fig. 3, A and B). Moreover, the extracellular concentrations of IL-16 and IL-1β protein correlated in a positive manner (Fig. 3C), whereas intracellular IL-16 (rMFI) in BAL CD8\(^+\) cells (P = 0.34, rho = 0.39) and extracellular IL-16 in cell-free BAL fluid (P = 0.61, rho = 0.15), respectively, did not display any statistically significant correlation with lung function [forced expiratory volume in 1 s (FEV\(_1\)) percentage of predicted, data not shown]. Extracellular TNF in BAL was not detected in 11 out of 12 chronic smokers and in 10 out of 11 never-smokers (data not shown). IL-4 and IFN-γ were not detectable in any BAL sample.

Samples from Occasional Smokers

Short-term exposure to tobacco smoke did not cause any substantial alteration of the concentration of extracellular IL-16 protein in BAL samples from occasional smokers (BAL 2) compared with preexposure values (BAL 1) or corresponding values after repeated bronchoscopy of never-smokers (Fig. 3D).
Samples from Blood CD8\(^+\) Cells Cultured In Vitro

**Time course for extracellular IL-16 protein.** Exposure to CSE did not cause any substantial increase in the concentration (picograms per milliliter; \(n = 8–11\)) of extracellular IL-16 protein until after 20 h of culture. For vehicle- and CSE-treated cells, respectively, the median (range) concentrations were as follows: 590 (231–1,769) and 390 (151–1,849) after 2.5 h; 390 (159–1,736) and 342 (136–1,297) after 5 h; 548 (167–787) and 544 (194–1,461) after 10 h; and, finally, 480 (139–675) and 714 (517–

![Image](image-url)

**Fig. 5.** Flow cytometry analysis of enriched human blood CD8\(^+\) cells cultured for 20 h in vitro. Shown are the tMFI for intracellular IL-16 protein (A), the percentage of CD8\(^+\) cells with intracellular IL-16 protein (B), and original and representative flow cytometry recordings (C). IgG1 PE served as a negative control. CD8\(^+\) cells were exposed to either water-soluble tobacco smoke components (CSE; \(\times 141\)) or vehicle solution (veh) and treated with aminophylline (AM; \(10^{-3}\) M) or glutathione (GL; \(10^{-2}\) M) or the corresponding vehicle solution. Stimulation (Stim.) with calcium ionophore (CI) in combination with PMA served as a positive control (pos). The results are presented as individual and median values (bold lines). *\(P < 0.05\) (Kruskal-Wallis followed by Mann-Whitney U test). FSC-H, forward scatter height; FL1-H, fluorescence 1 height.
The positive control (CI + PMA) was 696 (219–3,000) at 20 h.

Protein oxidation. The relative amount of oxidized proteins in CD8\(^+\) cells was markedly increased by CSE in three out of three paired experiments (Fig. 4, A and B). No unspecific binding occurred (Fig. 4B).

Intracellular IL-16 protein. Exposure to CSE markedly decreased intracellular IL-16 protein (rMFI) in blood CD8\(^+\) cells as well as the percentage of CD8\(^+\) cells with a positive signal for intracellular IL-16 (Fig. 5, A–C). Glutathione treatment fully reversed this process, whereas aminophylline did not. Neither drug exerted any substantial influence on intracellular IL-16 protein or on the fraction of IL-16\(^+\)CD8\(^+\) cells in the absence of CSE.

IL-16 mRNA. Exposure to CSE markedly decreased IL-16 mRNA in CD8\(^+\) cells (Fig. 6). Glutathione treatment prevented this decrease, whereas aminophylline treatment potentiated the CSE-induced effect.

Extracellular IL-16 protein and pharmacological interventions. Exposure to CSE markedly increased the concentration of extracellular IL-16 protein in conditioned medium from CD8\(^+\) cells; the (median with range) increase was 4-fold (3.8–12) compared with the negative (vehicle) control group (Fig. 7, A and B). Glutathione and aminophylline treatment almost completely attenuated this CSE-induced increase in IL-16 protein, in a concentration-dependent manner (Fig. 7, A and B). In contrast, treatment with cycloheximide, cyclosporin A, hydrocortisone, and terbutaline or with hydrocortisone and terbutaline in combination did not markedly affect the CSE-induced release of IL-16 protein (Table 2). None of the used drugs exerted any substantial influence on extracellular IL-16 protein in the absence of CSE.

Treatment with the caspase-3 inhibitor did not cause any clear inhibitory effect on the CSE-induced increase in extracellular IL-16 protein (picograms per milliliter; \(n = 9–11\)) at any concentration (Wilcoxon signed rank-test, \(P > 0.05\)). For CSE-stimulated cells, with and without vehicle and caspase-3 inhibitor treatment, respectively, the median (range) IL-16 concentrations were as follows: 253 (155–720) and 398 (165–1,192) at 10\(^{-4}\) M. In contrast, the caspase-3 inhibitor increased the concentration of extracellular IL-16 protein (picograms per milliliter; \(n = 8\)) in unstimulated CD8\(^+\) cells (Wilcoxon signed rank-test, \(P < 0.05\)). For vehicle- and anti-caspase-3-treated cells, respectively, the median (range) concentrations were as follows: 155–720 and 165–1,192 at 10\(^{-4}\) M.

Fig. 6. Levels of IL-16 mRNA normalized to a reference gene (HPRT) in human blood CD8\(^+\) cells cultured for 10 h in vitro and measured using real-time RT-PCR. The CD8\(^+\) cells were exposed to either water-soluble tobacco smoke components (CSE; •) or vehicle solution (veh) and treated with AM (10\(^{-3}\) M) or GL (10\(^{-2}\) M) or the corresponding vehicle solution. Stimulation with CI in combination with PMA served as a positive control. The results are presented as individual as well as median values (bold lines). * \(P < 0.05\) (Kruskal-Wallis followed by Mann-Whitney U test).

Fig. 7. Shown are concentrations of extracellular IL-16 protein measured by ELISA in conditioned medium from human blood CD8\(^+\) cells cultured for 20 h, exposed to either water-soluble tobacco smoke components (CSE; •) or vehicle solution (veh), and treated with GL (A; Spearman rank correlation, * \(P < 0.05\), \(\rho = -0.73\)), AM (B; Spearman rank correlation, * \(P < 0.05\), \(\rho = -0.64\)), or the corresponding vehicle solution in vitro. Stimulation with CI and PMA served as a positive control. The results are presented as individual as well as median values (bold lines).
Table 2. Concentration of extracellular IL-16 protein in conditioned medium from human blood CD8+ cells exposed for 20 h to water-soluble tobacco smoke components (CSE), with and without treatment with cycloheximide, cyclosporin A, hydrocortisone, terbutaline, or hydrocortisone and terbutaline in combination, as well as vehicle (Veh.) solution in vitro

<table>
<thead>
<tr>
<th>Drug</th>
<th>Veh.</th>
<th>Veh. + Drug</th>
<th>CSE 10⁻⁶ M</th>
<th>CSE 10⁻⁷ M</th>
<th>CSE 10⁻⁸ M</th>
<th>CSE 10⁻⁹ M</th>
<th>CD/PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide</td>
<td>69 (41–85)</td>
<td>94 (68–141)</td>
<td>524 (224–1,457)</td>
<td>516 (344–1,348)</td>
<td>510 (287–1,667)</td>
<td>408 (220–1,805)</td>
<td>340 (146–537)</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>71 (41–105)</td>
<td>86 (51–145)</td>
<td>563 (249–1,457)</td>
<td>821 (539–1,386)</td>
<td>763 (424–1,446)</td>
<td>604 (376–1,353)</td>
<td>337 (146–571)</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>76 (60–152)</td>
<td>78 (55–125)</td>
<td>484 (284–611)</td>
<td>391 (330–637)</td>
<td>389 (290–387)</td>
<td>357 (187–545)</td>
<td>557 (404–1,209)</td>
</tr>
</tbody>
</table>

Results are presented as medians with ranges. Stimulation with calcium ionophore (CI) in combination with PMA served as a positive control, n = 6. P > 0.05 for all treatments vs. controls (Kruskal-Wallis test).

**Cell activation.** Exposure to CSE led to a modest increase in the fraction of CD8+ cells expressing CD69 (Fig. 8A). Treatment with glutathione inhibited this increase in CD69, whereas aminophylline did not. Neither glutathione nor aminophylline had any pronounced effect on CD69 in the absence of CSE.

**Cell membrane integrity.** CSE increased the uptake of 7-AAD (Fig. 8B), which exceeded the increase observed in the positive control group. Nevertheless, CD8+ cells exposed to CSE had a side and forward scatter similar to that observed in negative controls, with a small CSE-related decrease in cell size only (Fig. 8C). Glutathione completely reversed the effect of CSE on 7-AAD, whereas aminophylline did not. Neither glutathione nor aminophylline exerted any substantial influence on 7-AAD in the absence of CSE (Fig. 8B).

**Apoptosis.** Exposure to CSE increased active caspase-3 in CD8+ cells in a modest but statistically significant manner (Fig. 8D). Glutathione treatment completely attenuated the effect of CSE. In contrast, aminophylline potentiated the CSE-induced increase in active caspase-3. Treatment with aminophylline, but not glutathione, also tended to increase caspase-3 in the absence of CSE.

**Cell proliferation.** Notably, CSE almost completely inhibited the uptake of radioactive thymidine in CD8+ cells (Fig. 9). Glutathione treatment partially prevented this effect, but aminophylline did not.

**DISCUSSION**

With the results of the current study, we forward evidence supporting the hypothesis that exposure to tobacco smoke depletes intracellular IL-16 protein and inhibits de novo production of IL-16 in CD8+ cells from human airways and blood while increasing extracellular IL-16 and that OFR are involved in mediating these effects. We also forward evidence that water-soluble components of tobacco smoke require a substantial period of time (i.e., ≥20 h) to produce their effects on the release of IL-16 protein into the extracellular compartment in human CD8+ cells.

Specifically, we found that chronic smokers have less intracellular IL-16 protein in the BAL CD8+ cells as well as less IL-16 mRNA in their BAL cell samples despite the simultaneous and clearly increased concentration of extracellular IL-16 protein in cell-free BAL fluid samples. Moreover, the increase in extracellular IL-16 was paralleled by an increase in the archetype proinflammatory cytokine IL-1β in the same compartment, and these two cytokines had a statistically significant, positive correlation. In contrast, we found no increase in extracellular IL-16 protein in BAL samples from occasional smokers after short-term exposure to tobacco smoke. By analogy, CSE caused a similar effect on human blood CD8+ cells cultured in vitro, cells that displayed reduced intracellular IL-16 protein and decreased IL-16 mRNA as well as a simultaneous increase in the concentration of extracellular IL-16 protein after CSE exposure. Moreover, CSE exerted a negative impact on several more generic markers of key cell functions in the human CD8+ cells. These markers of cell function included 7-AAD but not trypan blue exclusion, and this suggests functional damage but not disruption of cell membranes, the latter a phenomenon that would suggest cell necrosis if present. We also found that CSE exerted a weak impact on apoptosis, as indicated by active caspase-3 and a more clear, negative impact on proliferation, as indicated by uptake of radioactive thymidine in the blood CD8+ cells. However, we did observe a small increase in CD69, compatible with some increase in activity.

Interestingly, among the drugs tested, the OFR scavenger glutathione was the only one that countered all the described effects of CSE on blood CD8+ cells in vitro, and we also detected a clear CSE-induced increase in protein oxidation in the CD8+ cells cultured in vitro.

Importantly, the “big picture” is that our assessments of intracellular IL-16 protein with flow cytometry revealed a consistent pattern for the impact of tobacco smoke components on human CD8+ cells both in vivo and in vitro. In both cases, we detected a decrease in intracellular IL-16 protein in CD8+ cells. However, the induced decrease in the percentage of IL-16+CD8+ cells reached statistical significance only in vitro, probably due to the greater variability among the limited number of human subjects who were examined in vivo. This depletion of IL-16 protein by tobacco smoke components in vivo and in vitro underlines the pathogenic potential and the mechanistic significance of the previously demonstrated decrease in intracellular IL-16 protein in human palatine tonsils from chronic smokers (1).

In line with the previously published mechanistic link between IL-16 and IL-1β in human blood mononuclear cells cultured in vitro (24), we now show that the concentration of extracellular IL-1β correlates with IL-16 in BAL samples from chronic smokers. Indeed, we (1, 20) have earlier reported that chronic tobacco smoke causes an increase in extracellular IL-16 protein in BAL and now provide indirect evidence that...
repeated long-term exposure to tobacco smoke is required to establish this increase of IL-16 protein in the airways, since short-term exposure to tobacco smoke did not result in a corresponding increase in occasional smokers.

We do not believe that our current findings on IL-16 can be attributed to the uneven distribution of lung tumors or lung function in our study material. This is because, when analyzing individual subjects with and without tumors, we did not detect...
any tumor-related difference per se in IL-16 mRNA or in intracellular or extracellular IL-16 protein in BAL samples. It is also because neither intracellular nor extracellular IL-16 protein correlated with lung function (FEV₁ percentage of predicted) in chronic smokers.

Based on the current evidence, it can be speculated that chronic tobacco smoke exposure contributes to the local accumulation of CD4⁺ cells in the airways of chronic smokers with severe COPD. Hypothetically, the series of events leading to this type of CD4⁺ cell accumulation may include a transient release of IL-16 that occurs immediately when CD8⁺ cells enter the airways and become exposed to tobacco smoke components in high concentrations. Interestingly, there is now evidence that IL-16 preferentially recruits T regulatory cells, a group of suppressive CD4⁺ cells (25) and T regulatory cells may also dominate the airways of smokers (4, 34). If the referred evidence is correct, then it could mean that T regulatory cells are accumulated locally due to IL-16 and thereby contribute to the suppression of the adaptive arm of host defense locally in the airways.

We found that short-term exposure to tobacco smoke in occasional smokers does not increase extracellular IL-16 protein in BAL samples, whereas exposure to CSE does increase extracellular IL-16 protein in conditioned medium from blood CD8⁺ cells in vitro. These observations may seem contradictory from a mechanistic perspective, but it may very well be that the dose of water-soluble tobacco smoke components that we generated by using CSE in vitro can only be achieved in human airways in vivo by repeated, chronic exposure to tobacco smoke.

It is of mechanistic interest that neither the protein synthesis inhibitor cycloheximide nor the used caspase-3 inhibitor markedly inhibited the CSE-induced effect on extracellular IL-16 protein in blood CD8⁺ cells cultured in vitro. Given what is currently believed about the role for caspase-3 in transforming pro-IL-16 to active IL-16, these negative findings, together with the very modest increase of active caspase-3, are compatible with tobacco smoke components mainly releasing preformed IL-16 protein. Importantly, this is also mechanistically compatible with a simultaneous inhibition of de novo production of IL-16 protein, as shown in our current study.

We also observed that CSE causes an increase in the uptake of 7-AAD in blood CD8⁺ cells cultivated in vitro, suggesting that water-soluble tobacco smoke components damage the integrity of the cell membrane. In addition, we found that CSE exposure per se only causes a small decrease in side and forward scatter for blood CD8⁺ cells analyzed by flow cytometry, whereas CSE does not cause a reduced exclusion of trypan blue dye for the same CD8⁺ cells. Taken together, these observations argue that the human CD8⁺ cells maintain their fundamental structure and are alive even after exposure to high concentrations of water-soluble tobacco smoke components, even if not in optimum condition. These findings thus suggest an action of tobacco smoke components that is more delicate than mere cytotoxicity. At the same time, the CSE-induced increase in active caspase-3 in blood CD8⁺ cells in vitro suggests that a certain degree of apoptosis can be initiated by water-soluble tobacco smoke components. Moreover, because caspase-3 is believed to be required for the cleavage of pro-IL-16 into active IL-16 (43), and we here demonstrate a decrease in intracellular IL-16 caused by CSE, it seems feasible that the depleting effect of tobacco smoke components quantitatively exceeds the putatively minor facilitation of conversion of pro-IL-16 into the active IL-16.

It is known that the endogenous OFR scavenger glutathione is abundant in the epithelial lining fluid within the airways of healthy human subjects (7). It is therefore of interest that glutathione treatment more or less counteracted all measured effects caused by CSE in the human blood CD8⁺ cells investigated in our present study. Moreover, in the case of the CSE-induced increase of extracellular IL-16 protein in blood CD8⁺ cells, we found that the effect of glutathione treatment is concentration dependent. Importantly, we did detect a high degree of protein oxidation in blood CD8⁺ cells in vitro after stimulation with CSE, and, taken together, these observations...
are all compatible with OFR constituting key mediators of the effects of tobacco smoke components on human CD8+ cells. Clearly, these observations may have clinical implications, since OFR in tobacco smoke, as well as those formed by the inflammatory response per se, jointly increase the local burden of oxidants in the airways (11, 14).

We (1) have previously shown that CSE does not cause any pronounced increase in the T cell activation marker IL-2Ra, and we now show that CSE has a very modest effect on the T cell activation marker CD69, using a similar cell model (human blood T cells) in both cases. Together, these observations suggest that tobacco smoke components exert no strong, traditional activation of human lymphocytes, a conclusion that is also supported by the current lack of impact on extracellular IL-16 exerted by cyclosporine A, a classic immunosuppressant drug that acts as a calcineurin phosphatase inhibitor in T cells (2). Overall, these findings support the notion that the tobacco smoke-induced release of IL-16 protein does not require any substantial degree of cell activation.

We also revealed that the nonselective phosphodiesterase inhibitor aminophylline exerts a concentration-dependent and almost completely attenuating effect on the CSE-induced release of extracellular IL-16 protein in blood CD8+ cells in vitro. We find this is of mechanistic interest, since it implies that the effect of water-soluble tobacco smoke components on human CD8+ cells are mediated by endogenous phosphodiesterases as well as by OFR. However, unlike the OFR scavenger glutathione, aminophylline does not seem to protect the CD8+ cells from the additional CSE-induced effects. Moreover, we found that aminophylline potentiates the effect of CSE on active caspase-3 and IL-16 mRNA. Hence, it may be that nonselective phosphodiesterase inhibition is detrimental in terms of apoptosis and IL-16 transcription in CD8+ cells. Of additional mechanistic interest, we obtained evidence that unstimulated CD8+ cells constitutively release extracellular IL-16, and the observed negative effect of aminophylline on IL-16 mRNA may at least partly explain why intracellular IL-16 protein is further decreased by aminophylline in CD8+ cells after exposure to CSE. This would be fully in line with what has previously been published on the direct relationship between IL-16 mRNA and intracellular protein (31).

Given current clinical practice in the treatment of COPD, it should be emphasized that our study forwards no evidence that treatment with a glucocorticoid receptor agonist and/or a β-adrenoceptor agonist can prevent the IL-16 release caused by the water-soluble components of tobacco smoke in human CD8+ cells. These experimental observations are also compatible with the clinical experience that neither of these drug classes has been proven to substantially alter the clinical progression of COPD (6, 23, 30).

Finally, even though we cannot rule out IL-16 sources other than CD8+ cells, it is worth noting that we were unable to detect such “alternative” sources in bronchial biopsy samples harvested from the current study population using immunostaining protocols previously proven to identify IL-16 in palatine tonsil tissue (1).

In conclusion, our current translational study provides novel evidence that exposure to tobacco smoke exerts a functional impact on human airway and body CD8+ cells. This exposure probably depletes intracellular IL-16 protein and inhibits de novo production of this CD4 chemoattractant as well as causes an increase in extracellular IL-16 protein. In addition, exposure to tobacco smoke may cause detrimental effects on additional key cellular functions, and OFR stand out as important mediators of these effects. Given what we show in this study, tobacco smoke bears the potential to distort the function of the adaptive arm of host defense.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s). No direct or indirect financial support was obtained from the tobacco industry.

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