Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells

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Summary Chronic obstructive pulmonary disease (COPD) is a disease primarily caused by cigarette smoking, which in turn has been shown to affect the susceptibility to and progression of airway infections. The question addressed in this study was how components from cigarette smoke could affect the defence mechanisms of T-cells and epithelial cells, and thereby contribute to the development of the COPD pathology. T-cells and monocytes were isolated from buffycoats from healthy donors and T-cell responses studied in response to cigarette smoke extract (CSE). Activation level (CD25 expression), proliferation (BrdU incorporation) and intracellular expression of the cytotoxic markers granzyme-b and TIA-1 were determined using flowcytometry. Normal human bronchial epithelial cells were obtained from Cambrex and differentiated in air–liquid interface cultures. After exposure to CSE barrier function (trans-epithelial electric resistance, TEER), MUC5AC and interleukin-8 production were measured. T-cell activation, proliferation and expression of the cytotoxic proteins granzyme-b and TIA-1 were significantly reduced in response to 0.5–1% of CSE. The epithelial cells were more resistant to CSE and responded at doses 20 times higher than T-cells. The expression of interleukin-8 and MUC5AC was significantly increased after exposure to 15% and 30% CSE and TEER was largely unaffected at 30% CSE but clearly reduced at 40% CSE. This study shows that mechanisms, in both T-cells and airway epithelial cells, involved in the defence against infectious agents are modulated by CSE.

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Background

In chronic obstructive pulmonary disease (COPD) and chronic bronchitis, two smoke related diseases, exacerbations are often triggered by respiratory infections. In addition, these patients often show exacerbations are often triggered by respiratory infections. In the lower respiratory tract, alveolar macrophages are the key cells to keep the environment sterile with the help from, among others, T-cells. This bacterial colonization has been shown to persist between exacerbations in the form of bio films or even intracellular within the epithelial cells. It has been shown that also asymptomatic smokers suffer from respiratory infections more frequently than non-smokers and studies on mice have shown that the recovery from infections and the clearance of virus and bacteria is impaired in response to nicotine. This raises questions about whether cigarette smoke has the ability to alter defence mechanisms of the lung.

The defence against microbial and viral infections in the respiratory tract is provided by a number of different mechanisms. In the central airways the epithelial lining is ciliated and covered with a protective mucus layer able to transport particles and microorganisms away from the central airways by mucociliary clearance. This epithelial barrier constitutes the first line of defence. Interspersed between the epithelial cells, mucosal T-cells are located as a specific defence against infectious agents that are penetrating the mucosal barrier. Further into the tissue, in the lamina propria, macrophages, neutrophils and T-cells provide both innate and adaptive immune responses to help clear infections. In the lower respiratory tract, alveolar macrophages are the key cells to keep the environment sterile with the help from, among others, T-cells. Together these cells constitute the normal defence of the lung, and have been shown to differ in number and/or appearance in smoke related diseases. Studies on bronchoalveolar lavage and biopsies from smokers with chronic bronchitis or COPD have revealed an increased number of CD8+ T-cells in the lungs of smokers as compared to non-smokers. Immunohistochemical studies of the bronchial epithelium in COPD patients have revealed a hypersecretory phenotype, with increased production of airway mucins as well as goblet cell hyperplasia. In more distal areas of the bronchial tree, where mucin production is not normally seen, goblet cell metaplasia has been demonstrated. In addition, increased levels of proinflammatory cytokines like interleukin-8 (IL-8), have been demonstrated in sputum and bronchoalveolar lavage fluid of COPD patients and probably accounts for the large influx of neutrophils seen in the lungs of these patients.

In the present study, T-cells and epithelial cells were investigated with the hypothesis that both T-cell and epithelial functions, involved in the defence against infectious agents, could be altered by the exposure to cigarette smoke components. Both cell types were stimulated in vitro with an aqueous cigarette smoke extract (CSE). Proliferation, activation and cytotoxic potential of the T-cells as well as barrier integrity, MUC5AC and IL-8 production of airway epithelial cells grown in air–liquid interface (ALI) cultures were studied.

Materials and methods

Cigarette smoke extract preparation

CSE was prepared in a way similar to what have been published previously. Smoke from two filterless Kentucky research cigarettes, 1R4F containing 11 mg tar and 0.8 mg nicotine, was bubbled through 50 ml of cell culture medium (for T-cells RPMI-1640 and for epithelial cells BEBM further described below) at a speed of 5 min per cigarette. The CSE was filtered through a 0.2 μm sterile filter (Corning, New York, USA) before addition to cell cultures. The CSE was used within 20 min of preparation to avoid breakdown of substances in the extract and evaporation of volatile components.

T-cell and monocyte preparation

Peripheral blood mononuclear cells (PBMCs), were isolated from buffycoats from healthy blood donors (Blood Centre, Lund University Hospital) by gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Sweden) according to the manufacturer’s recommendations. Total CD3+ T-cell populations, CD8+ T-cells and monocytes were extracted from PBMCs by positive selection on MACS beads directed against CD3, CD8 and CD14, respectively, using an Automacs system according to the manufactures instructions (Miltenyi biotec, Germany). The purity of the cells was checked by flowcytometry analysis for CD3, CD8, CD14 and CD16 using a FACS Calibur instrument from Becton Dickinson and the Cellquest Pro analysis software. The median values for purity of cell populations extracted from PBMCs using MACS beads were for CD3+ T-cells 97.5% (range: 90–98%), CD8+ T-cells 98% (range: 97–98%) and CD14+ cells 90% (range: 89–93%).

Culturing of T-cells and stimulation with CSE

Two types of T-cell cultures were set up; cocultures containing CD8+ T-cells and monocytes,
at a 1:1 ratio, and mono-cultures containing only CD3+ T-cells. All cultures were preformed in 24 well plates coated with anti-CD3 and supplemented with 5 μg/ml of soluable anti-CD28 (BD Pharmingen, San Diego, USA). Each well contained a total of one million cells, in co-cultures 0.5 millions of CD8+ T-cells and 0.5 millions of monocytes and in mono-cultures one million CD3+ T-cells. Cells were cultured in RPMI 1640 with glutamax-1 (a more stable glutamine derivate) and 25 mM HEPES (Invitrogen/Gibco, Paisley, UK) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin (Invitrogen/Gibco, Paisley, UK). CSE was added to the cultures at the time of the seeding in doses 0–1%. Incubation was performed at 5% CO₂, 95% humidity in a tissue culture incubator.

BrdU incorporation and Flowcytometry

On day 3 cultures for proliferation measurements were pulsed with 1 mM bromodeoxyuridine, BrdU (BD Pharmingen, San Diego, USA) and incubated for 2 h before harvest. Cells in mono-cultures were stained with antibodies against CD4 and CD8 (DAKO, Glostrup, Denmark) in order to monitor changes in the individual cell populations, and cells in the co-cultures were stained with antibodies against CD8 before they were all fixed, permeabilized and DNase treated. The cells were then stained for incorporated BrdU, using a monoclonal BrdU antibody and a matched isotype control (BD Pharmingen, San Diego, USA), and analysed on a FACS Calibur. Cultures not used for proliferation measurements were stained with antibodies against CD25, CD4 and CD8 (DAKO, Glostrup, Denmark) on the surface and granzyme-b (Serotec, Oxford, UK) and TIA-1 (Immunotech, Marseille, France) intracellular after permeabilization with Leucoperm (Serotec, Oxford UK). Matched isotype controls (DAKO, Glostrup, Denmark) were used to assure specificity of the positive antibody staining. In initial experiments T-cell viability and live gate settings in the flowcytometry analysis was evaluated using FITC labeled Annexin V staining (Alexis Corporation, Lausen, Switzerland). All FACS analysis was performed on a FACS Calibur instrument (BD Biosciences) using the Cellquest Pro software.

Epithelial cell culture

Primary normal human bronchial epithelial cells (NHBE) (donor NHBE 4892) were commercially obtained from Cambrex (East Rutherford, NJ, USA) at passage one. The cells were cultured in Bronchial Epithelial Growth Medium (BEGM) consisting of Bronchial Epithelial Basal Medium (BEBM) and all supplements, except antibiotics, according to the vendor’s recommendations (Cambrex, East Rutherford, NJ, USA). Cells were expanded in T75 flasks and frozen in aliquots at passage two. The cells were differentiated in an ALI culture system following the protocol of Gray et al. Briefly, the cells were seeded on Vitrogen coated 12 well Transwell clear insert plates (Corning, Acron, MA, USA) at a density of 40,000 cells/cm² in 1.5 ml BEGM basolaterally and 0.5 ml BEGM apically. Until reaching confluency (typically at day 4) cells were maintained in BEGM. The ALI was established by removing the apical medium and replacing the basolateral medium with ALI medium made up from Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen/Gibco, Paisely, UK) and BEBM in a ratio 1:1, supplemented with 0.4% bovine pituitary extract, 5 μg/ml insulin, 75 ng/ml hydrocortisone, 10 μg/ml transferrin, 6.5 ng/ml T3, 0.5 μg/ml epinephrine, 0.5 μg/ml epidermal growth factor, 15 μg/ml retinoic acid and 1.5 μg/ml BSA (final concentrations). The basolateral medium was changed daily during weekdays. Excess mucus was removed by rinsing the apical surface of the cells with 37 °C DMEM. Following approximately 14 days in ALI culture ciliated cells appeared. Cells were cultured for up to 10 weeks and used for experiments. For histological evaluation cultures were fixed in 4% paraformaldehyde and embedded into paraffin. Sections were stained with haematoxylin following standard protocols.

CSE exposure to epithelial cells

ALI cultures (age of culture indicated within each experiment) were starved for 24 h in BEBM medium containing 1% insulin/transferrin/selenin (ITS) solution (Sigma-Aldrich, UK) and 0.1% BSA prior to the experiments. Cells were apically rinsed to remove excess mucus and apically exposed to indicate concentrations of CSE in 300 μl BEBM for 30 h.

Assessment of barrier function in epithelial cells

Development of trans-epithelial electrical resistance (TEER) was monitored using chopstick electrodes (Millipore, Billerica, MA, USA) as a measurement of tight junctional barrier formation. BEBM (300 μl) were applied to the apical compartment and TEER was measured in at least two different locations of the culture. Only cultures with a TEER value of ≥ 800 Ω cm² were used in the CSE exposure experiments. ALI cultures
differentiated for 21 days were exposed to 5–40% CSE as described above and changes in TEER were measured.

**Determination of epithelial IL-8 release**

ALI cultures differentiated for 10–12 weeks were exposed to 15% and 30% of CSE as described above. Initial experiments had shown that lower concentrations, 5–10%, had no substantial effect on release of IL-8 (data not shown). The concentration of basolaterally released IL-8 after CSE stimulation was determined using a commercial IL-8 ELISA according to the vendor’s recommendations (R&D Systems, Minneapolis, MN, USA) and analysed using a Spectramax plus spectrophotometer (Molecular devices, Sunnyvale, CA, USA).

**Determination of MUC5AC secretion**

Apical secretion of MUC5AC was assessed using a modification of the ELISA protocol of Takeyama et al.18 Briefly the apical surface of cultures was washed with 500 µl 37 °C DMEM and cells stimulated with 15–30% of CSE as described above. After 30 h of CSE stimulation the apical medium was collected, centrifuged (1600 rpm/10 min/4 °C) and stored at −80 °C until analysis. For MUC5AC ELISA 70 µl of apical sample was mixed with 30 µl 0.05 M carbonate buffer and dried down on a RIA plate (Corning, Acron, MA, USA) overnight at 37 °C. MUC5AC protein was detected using the monoclonal antibody 45M1 (Labvision, Freemont, CA, USA) and an HRP-coupled goat-anti-mouse IgG secondary antibody (Sigma, St. Louis, MO, USA) using colorimetric detection. Plates were analysed using a Spectramax plus photometer (Molecular devices, Sunnyvale, CA, USA).

**Statistics**

Data were analysed using Wilcoxon Mann–Whitney U-test using the Astute package for Excel (DDU software, Leeds, UK). Differences between groups were considered significant at $P \leq 0.05$.

**Results**

**T-cell viability**

T-cell viability in response to CSE was assessed as percent of cells in a live gate of the flowcytometry analysis. The live gate was in initial experiments defined as an Annexin-V negative population as shown in Fig. 1. The viability of the T-cells exposed to 0.1% and 0.5% CSE did not significantly differ from that of cells grown in the absence of CSE. In cells exposed to 1% CSE the viability was lower than in unstimulated cells ($P < 0.05$) 69% (range: 45–72%)

![Figure 1](image)

**Figure 1** Live gating in flowcytometry analysis. The live cell gate in the flowcytometer was set as an Annexin-V negative population. The scatter plots show forward and side scatter of T-cells in co-cultures. R1 is the live gate and R2 is the characteristic Annexin-V positive cell population. The histogram shows the negative Annexin-V staining of the live gate.
and 76% (range: 72–85%), respectively, in co-cultures and 67% (range: 57–81%) and 84% (range: 83–90%), respectively, in mono-cultures. Only cells in the live gate were used in the flowcytometry analysis of activation, proliferation and cytotoxic markers.

**T-cell activation and proliferation**

The first experiments addressed the potential effects of CSE on the most basic T-cell functions such as general activation through the CD3/TcR complex resulting in upregulation of the high affinity IL-2 receptor (CD25) and proliferation. As expected, T-cells grown in the presence of monocytes had a statistically significant higher expression of CD25 compared to T-cells grown in the absence of monocytes (Fig. 2). Addition of CSE resulted in a significant reduction \((P < 0.05)\) in both the degree of activation (CD25 expression) and proliferation (BrdU incorporation) of the T-cells. Addition of 0.5% CSE was enough to induce a significant reduction in T-cell activation both in the presence and absence of monocytes. The proliferation was significantly reduced by adding 0.5% CSE to T-cells stimulated in the absence of monocytes and by 1% CSE for T-cells in cocultures with monocytes \((P < 0.05)\) (Fig. 3).

**T-cell cytotoxicity**

Next set of experiments was designed to address effects of CSE on the cytotoxic potential of the T-cells. The expression of the two cytotoxicity markers granzyme-b and TIA-1 was significantly reduced in response to CSE. A significant reduction of granzyme-b was detected at 0.5% of CSE in both mono-cultures \((P < 0.05)\) and co-cultures \((P < 0.05)\) (Fig. 4). TIA-1 was significantly reduced in CD4+ cells in mono-cultures at 0.5% of CSE \((P < 0.05)\), while for CD8+ T-cells in the same cultures the reduction of TIA-1 was not statistically significant. In CD8+ T-cells grown in the presence of monocytes, however, there was a significant decrease in TIA-1 at 0.5% of CSE \((P < 0.05)\) (Fig. 5).

**Epithelial cell differentiation**

ALI culturing of primary human epithelial cells induced differentiation into basal, ciliated and secretory (goblet) cells (Fig. 6). The morphological differentiation was accompanied by the development of a substantial TEER from day 13 in ALI culture and peaked at 1665 Ω cm\(^2\) around day 30.

**Epithelial responses to CSE exposure**

Exposure of differentiated ALI cultures (cultured 21 days in ALI) to CSE concentrations up to 30% for 30 h evoked no clear changes in TEER. However, 40% of CSE resulted in a drastic reduction of the resistance (Fig. 7). ALI cultures (cultured 10–12 weeks in ALI) exposed to 15% and 30% of CSE responded with a significant and dose dependent increase in basolateral IL-8 release with median values of 159% and 238% of control, respectively \((P < 0.05)\) (Fig. 8). Similarly, the apical MUC5AC secretion was significantly increased in response to 15% and 30% of CSE with median values of 190% and 246% of...
Figures 3 and 4 T-cell proliferation in response to CSE. The proliferation of T-cells, measured as incorporation of BrdU, was significantly reduced in the presence of CSE. A significant reduction in proliferation was found at a CSE concentration of 0.5% or more for T-cells grown in the absence of monocytes (P<0.05) and at 1% for T-cells grown in the presence of monocytes (P<0.05). Bars designating the median values.

Figures 4 and 5 T-cell expression of cytotoxic markers in response to CSE. The cytotoxicity markers TIA-1 and granzyme-b were both negatively affected by CSE. The granzyme-b expression was significantly reduced in both the CD4+ and the CD8+ T-cells in the T-cell mono-cultures at a CSE concentration of 0.5% or more (P<0.05) (Fig. 4 (top)) while the TIA-1 expression in the same cells was significantly reduced in the CD4+ cells, at a CSE concentration of 0.5% or more (P<0.05), but not in the CD8+ cells (Fig. 5 (bottom)). However, grown in the presence of monocytes the expression of TIA-1 in CD8+ T-cells was significantly reduced at a CSE concentration of 0.5% or more (P<0.05) (Fig. 5), as was the granzyme-b expression (P<0.05) (Fig. 4). Bars designating the median values.
control, respectively \( (P<0.05) \) (Fig. 9). At CSE concentrations up to 30% ciliary beat remained visible and no dead cells or disruption of the cell layer were visible (data not shown). No IL-8 or MUC5AC measurements were made in cultures exposed to 40% CSE due to the clear cytotoxic effects on the cells that was recorded as outlined above.

**Discussion**

A number of different pathological changes contribute to the development of COPD. Proteolytic tissue degradation possibly due to an imbalance in the protease/anti-protease balance, may result in emphysematous changes of the peripheral lung tissue, and a decline in lung function. In other cases the obstruction seem more related to severe bronchitis and bronchiolitis accompanied by occlusion of small airways due to mucus plugging and tissue remodelling, and in many cases combinations of these events contribute to the disease progression.  

A third factor, possibly contributing to progression of the disease, is reoccurring exacerbations, often triggered by airway infections. The present study focused on possible effects of components in cigarette smoke on the defence mechanisms against airway infections in T-cells and epithelial cells.

Since T-cells from human airways are hard to extract in numbers sufficient for functional analysis, the T-cell responses to CSE in the present study were studied using cells from peripheral blood. The
cells were cultured in vitro and stimulated with CSE. Already at very low doses (0.5–1%) T-cells responded with a reduction in CD25 expression, proliferation and production of cytotoxic granule proteins granzyme-b and TIA-1. This was seen in both CD4+ and CD8+ T-cells even when the cells were fully stimulated, as shown in co-culture experiments with CD8+ T-cells and monocytes. These results indicate that T-cells are very susceptible to cigarette smoke in a way that could impair their cytotoxic capacity to fight infections.

The epithelial cells, here represented by normal bronchial epithelial cells grown in ALI, were much more resistant to the CSE and responded with an increased production of both MUC5AC and IL-8 at doses up to 30% CSE. Not until 40% CSE was added to the cells did the trans-epithelial resistance drop, reflecting a breakdown of the epithelial integrity. The epithelial cells hence show a strong resistance to cigarette smoke and also respond in a way that could affect their defence mechanisms. The increased mucous-layer provides good protection against particulate irritants, but also a suitable milieu for colonization with bacteria which could promote infections. The increased production of the neutrophil chemo-attractant IL-8, contributes to an increased innate host defence against bacterial and viral infections, but much like the mucus production, an increased number of neutrophils in the submucosa could also promote epithelial cell-damage through release of proteases and reactive oxygen species by these neutrophils, thereby increasing susceptibility to the same infections.

To understand the reasons for the large differences seen between T-cell and epithelial cell responses further analysis on active components in CSE responsible for these effects has to be made. Studies on T-cell proliferation in response to components in cigarette smoke have shown results similar to the present study. Geiselhart et al. studied T-cell proliferation in response to tar components and found that p-benzoquinone and hydroquinone affected IL-2 dependent activation and proliferation respectively in a negative way. In another study by Geng et al., rats were exposed to nicotine via mini osmotic pumps and T-cell proliferation was shown to be negatively affected by nicotine at doses similar to those of cigarette smokers. They also showed that TCR-induced T-cell activation was decreased after nicotine exposure. In two different studies where epithelial cells were exposed to mainstream cigarette smoke and acrolein, respectively, breakdown of the epithelium, shown as decline in TEER and increase in passive transport of 14C-mannitol, was reported, indicating that volatile components of the cigarette smoke could influence the epithelial responses.

The culture conditions as well as different compositions and concentrations of CSE used in in vitro studies will of course influence the results. To the authors knowledge only one study, made by Andersson et al., has been published on T-cell responses to CSE so far. In that study peripheral blood T-cells were grown in culture conditions similar to the ones in the present study, with the exception of CSE stimulation performed during 20 h instead of 72 h as done in the present study. The readouts in the study by Andersson et al. were different from the ones in the present study making the data on CD25 expression, proliferation and expression of granzyme-b and TIA-1 presented here the first of their kind. With respect to epithelial cells, these cells are reported to be more susceptible to CSE when studied in submersed cultures than are cells in ALI cultures (Möller, unpublished observations). The production of MUC5AC and IL-8 in ALI cultures in response to CSE presented in this study has to the authors knowledge not been published before. These data support studies made on the human epithelial cell line NCI-H292, which showed similar results with increased MUC5AC production in dose response to CSE and IL-8 production in response to CSE.

In the present study, T-cells and epithelial cells were exposed to CSE prepared in the same way making it easier to compare the results from the different cell types. This is otherwise a problem when comparing in vitro studies on cigarette smoke exposure. The use of different definitions of CSE dosage makes it hard to compare results from different studies as some groups define doses as % CSE others as cigarettes per millilitre and yet others based on absorbance. Also, other factors like the choice and number of cigarettes, volume of medium and combustion rate may influence the composition and effects of CSE and make comparisons hard. Standardization of cigarettes and smoke extraction protocols would be useful tools for future studies.

Even though CSE stimulation is an in vitro based system that should be interpreted with caution, some clinical aspects could be discussed from the study presented here. The CSE effect on T-cell CD25 expression and proliferation was not surprising since many articles have been published on T-cell unresponsiveness in BAL from smokers. The reduced production of cytotoxic granule proteins TIA-1 and granzyme-b, which are both involved in...
DNA damage and apoptosis of target cells, was less predictable since another cytotoxic protein, perforin, has been shown to be up regulated in sputum CD8+ T-cells of smokers when compared to non-smokers. If the effect of cigarette smoke components on T-cell production of granzyme-b and TIA-1 shown here reflect the in vivo situation, T-cells exposed to cigarette smoke would be impaired in their cytotoxic function, which could lead to an inferior defence against airway infections. The decreased effector functions could explain how the relatively high numbers of T-cells that have been reported to be present in the lungs of COPD patients and smokers can coincide with frequent airway infections and reduced viral clearance.

On similar note, the ALI cultures of primary human epithelial cells used in this study could provide a relevant cell model of the tracheal epithelium, and the up regulation of MUC5AC and IL-8 production have been manifested in tissue and sputum/lavage fluids respectively, in smokers and COPD patients. Our data suggest that cigarette smoke could be an important stimulus responsible for these changes. In addition, chronic exposure to cigarette smoke and the resulting changes in quantity and composition of the mucus layer, resulting in impaired mucociliary clearance of pathogens and changes in the hydration state of the mucus, could promote pathogen colonization in the airway lumen.

Taken together, this study shows that mechanisms, in both T-cells and epithelial cells, involved in defence against infections can be altered by cigarette smoke components. Since exacerbations in COPD patients are often triggered by respiratory infections, knowledge of how cigarette smoking affects the likelihood of contracting and coping with such infections is of great importance.

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References


