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Cigarette smoke induces the release of CXCL-8 from human bronchial epithelial cells via TLRs and induction of the inflammasome

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ABSTRACT

COPD is a chronic airway disease associated with inflammation and cigarette smoking. Airway epithelial cells are the first cells exposed to cigarette smoke (CS) and can release CXCL-8 and IL-1β. These cytokines are involved in acute and chronic inflammatory processes in COPD. The aim of this study was to investigate whether toll-like receptors (TLRs) located in/on epithelial cells were involved in cigarette smoke-induced cytokine production. Here we demonstrate that CS induces the release of CXCL-8 and IL-1β from human bronchial epithelial cells (HBE-14o). CS-induced CXCL-8 production was inhibited by an antibody against TLR4 and by inhibitory ODN suggesting the involvement of TLR4 and TLR9. In addition, exposure of HBE-14o cells to TLR4 or TLR9 ligands resulted in the release of CXCL-8 and IL1β. TLR4 and also TLR9 were present on the cell surface and the expression of both receptors decreased after CS exposure. The molecular mechanism of the CS-induced CXCL-8 production by the epithelial cells was further investigated. It was found that P2X7 receptors and reactive oxygen species were involved. Interestingly, the inflammasome activator monosodium urate crystals (MSU) induced the release of CXCL-8 and IL-1β and the caspase-1 inhibitor Z-VADDCB suppressed the CS-induced release of CXCL-8. In addition, CS, CpGODN, lipopolysaccharide and MSU all increased the expression of caspase-1 and IL-1β. In conclusion, our results demonstrate that CS releases CXCL-8 from HBE-14o cells via TLR4 and TLR9 and inflammasome activation. Therefore, inflammasome signaling in airway epithelial cells may play an important role in pathogenesis of diseases like COPD.

1. Introduction

Cytokines and chemokines are important mediators in lung defense and inflammation [20]. Exposure to cigarette smoke, noxious particles or gases can activate an inflammatory cascade in the airways resulting in the production of a number of potent cytokines and chemokines [49]. The airway epithelium is the first barrier for pathogens and particles and is also a target for factors released by infiltrating inflammatory cells [43]. Furthermore, airway epithelial cells are themselves potent producers of inflammatory mediators by which the inflammation can be initiated and sustained within the airways. Cigarette smoking and thereby cigarette smoke is a major risk factor in the pathogenesis of COPD [17]. Several inflammatory cells and their mediators participate in the inflammatory response in COPD. These mediators play a role in the induction of neutrophil infiltration (CXCL-8) and chronic inflammation (IL-1β) thereby leading to tissue destruction [4,5]. CXCL-8 is rapidly produced by different cell types upon incubation with inflammatory stimuli such as TNF-α, LPS and cigarette smoke and is one of the most potent neutrophil chemoattractants in human tissue [14,19,30]. IL-1β induces the release of GM-CSF which leads to the activation and increased survival of monocytes/macrophages and enhanced oxidative burst in the lungs, thus maintaining and prolonging inflammatory reactions [48]. IL-1β is secreted after activation of inflammasome signaling which is triggered by signal transduction via TLRs and purinergic receptors [9,28]. Apoptosis associated speck-like protein, containing a caspase recruit domain (ASC) and pro-caspase-1, form a multimeric cytosolic molecular complex known as the NALP3 inflammasome [35]. Activation of cytokine receptors or pattern recognition receptors such as TLRs leads to the induction of pro-IL-1β, NALP3 and activation of caspase-1 [37,38] resulting in the proteolitic release of IL-1β family cytokines [35].

Abbreviations: ATP, Adenosine-5′-triphosphate; COPD, Chronic obstructive pulmonary disease; CSE, Cigarette smoke extracts; CXCL-8, Interleukin-8; ELISA, Enzyme-linked immunosorbent assay; LPS, Lipopolysaccharide; MSU, Monosodium urate crystals; NALP3, NACHT, LRR and PYD domain-containing protein 3; NAC, N-acetyl-l-cystein; NF-κB, Nuclear factor-κB; ODN, Oligodeoxynucleotides; PBS, Phosphate-buffered saline; ROS, Reactive oxygen species; TLR4, Toll-like receptor 4; TLR9, Toll-like receptor 9; Z-VAD-DCB, Z-Val-Ala-Asp-dichlorobenzoate

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TLRs are pathogen-associated molecular pattern receptors for diverse microbial-derived molecules that are expressed predominantly on innate immune cells [2]. To date, eleven TLR family members have been identified in the human genome [1]. TLR4 and TLR9 play a critical role in lung and respiratory tract diseases [18,46,50,51]. TLR9 is localized in intracellular compartments, and recognizes viral and bacterial DNA that contains unmethylated CpG dinucleotides in certain base contexts [15,44]. Ewaschuk et al. demonstrated expression of TLR9 on the surface of intestinal epithelial cells [12], but the presence of TLR9 on the surface of other epithelial cell types is not well described yet.

Tobacco is known to be contaminated with microbes from which the cell wall components, DNA or RNA might activate TLRs [33]. However, it cannot be excluded that other components in cigarette smoke or the genetic material of the tobacco leaves contribute to activation of TLRs. Recently, we have shown that cigarette smoke activates TLR4 [19,40] and TLR9 signal transduction [26] in different types of (human) immune cells. Moreover, we have reported that cigarette smoke induces the release of adenosine-5′-triphosphate (ATP) and activates purinergic receptor P2X7 signal transduction pathways of human neutrophils [27]. The aims of the present study were to evaluate whether cigarette smoke exposure: (1) induces the release of specific chemokines and cytokines that are crucially involved in the pathogenesis of COPD (2) alters the expression of TLRs and (3) affects the activation of inflammasome in human epithelial cells.

2. Materials and methods

2.1. Reagents

LPS (*Escherichia coli* 055.B5), N-acetylcysteine (NAC), curcumin, chloroquine, propidium iodide and L-NAME were purchased from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands). RPMI 1640, Tyrode's buffer, fetal bovine serum, and non-essential amino acids were purchased from GibCo BRL Life Technologies (GIBCO-BRL-Invitrogen Corporation, Carlsbad, CA, USA). Rabbit polyclonal antibodies against IL-1β, caspase-1 and GAPDH were obtained from Cell signaling (Bioke, Leiden, The Netherlands) and Stressgen (Enzo Life Sciences BV, Zandhoven, Belgium), respectively. AZ11645373 (as P2X7 receptor antagonist) and ZVAD-DCB (as a caspase-1 inhibitor) were purchased from Tocris Bioscience, (Missouri, USA) and Bachem Distribution Services GmbH (Weil am Rhein Germany), respectively. The precision protein standards and PVDF membrane were purchased from Bio-Rad (Bio-Rad Laboratories, Veenendaal, The Netherlands). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG were purchased from Dako Diagnostics (Dako B.V. Heverlee, Belgium). CpGODN 2395 stimulatory oligonucleotide, negative blocking control inhibitory ODN without the CpG motif (inhibitory ODN with sequence 5′-tttagggttagggttagggtt agg g-3, ODN control (ODN 2395 control with sequence 5′-tgctgcttttgggggggccccc-3′) and MSU (as a stimulator for inflammasome signaling) were purchased from Invivogen (Cayla-InvivoGen Europe, Toulouse, France).

Functional neutralizing anti-TLR4 was purchased from eBioscience (NatuTec GmbH, Frankfurt, Germany).

2.2. Airway bronchial epithelial cell culture

The 16 HBE-14o human bronchial epithelial cell line was kindly provided by Dr. Gruenert (University of California at San Francisco, San Francisco, CA) [6]. Cells were grown in a MEM culture medium containing 0.292 g/L L-glutamine, 1 g/L glucose, 2.2 g/L $NaHCO_3$ and 10% fetal bovine serum (FBS) and antibiotics (complete culture medium) mixed with Vitrogen 100 (3 mg/ml Collagen I, bovine) and human fibronectin (1 mg/ml) in 75 cell culture flasks. At confluence, cells were washed with PBS and quiesced overnight in serum-free MEM-ITS [basal MEM containing 2 mM L-glutamine and antibiotic/antimycotic (100 U/ml penicillin G, 100 mg/ml streptomycin, 250 ng/ml amphotericin B (antibiotic–antimycotic liquid supplement (Invitrogen) supplemented with 10 mg/ml bovine insulin, 5.5 mg/ml human transferrin and 5 ng/ml sodium selenite (Sigma). Before each experiment, cells were washed by adding 500 μl per well of PBS/saline, and thereafter 250 μl of fresh, serum-free MEM-ITS was added to each well.

2.3. Preparation of cigarette smoke extracts (CSE)

CSE was prepared as described previously [19]. CSE was generated by the burning of commercially available Lucky Strike cigarettes without filter (British–American Tobacco, Groningen, The Netherlands), using the TE-10z smoking machine (Teague Enterprises, Davis, CA, USA) which is programmed to smoke cigarettes according to the Federal Trade Commission protocol (35-ml puff volume drawn for 2 s, once per minute). The machine produced main- and side stream smoke from one cigarette through a 5-ml culture medium (RPMI without phenol red). The absorbance was then measured using a spectophotometer and the media were standardized to a standard curve of CSM concentration against absorbance at 320 nm. The pH of the resultant extract was titrated to pH 7.4 and diluted with medium. This concentration (optical density [OD] = 4.0) was serially diluted with untreated media to 0.03, 0.06 and 0.12 OD and applied to the cells. Except for the dose–response and viability test, CSM at a concentration of 0.06 OD was selected for use in all experiments. Toxic concentrations of CSE were detected by performing different toxicological assays (as described in Cell viability assay section).

2.4. Pharmacological interaction of inflammasome and ROS/NO pathways

Cells (1×10^6/ml) were preincubated with AZ11645373 (100 nM) [23] *or* ZVAD-DCB (10 μM) [3] for 30 min before incubation with CSE. In another experiment cells were preincubated for 30 min with L-NAME (10 mM) (as a blocker for generation of NO) and NAC (10 mM) (as a ROS scavenger). In all conditions after preincubation cells were stimulated with CSE (1.5%) for 16 h, the supernatant harvested, and the level of CXCL-8 determined by ELISA.

2.5. Stimulation of cells

Cells (1×10^6 cells/ml) were incubated with various concentrations of CSE (0.75–3%) for 16 h. Cells were simultaneously activated with CpGODN type C (3 μM) or control ODN (5 mM), LPS (1 μg/ml), or MSU (0.2 mg/ml) (as an activator for inflammasome signaling) for 16 h. The level of cytokines was measured in the supernatant of harvested cells. Next, cells were preincubated with anti-human TLR4 (clone HTA125) or mouse IgG2a isotype control (20 μg/ml) or inhibitory ODN (10 mM) (Invivogen) for 30 min or 1 h at 37 °C to study the involvement of anti-TLRs on CXCL-8 production. Lastly, cells were stimulated with CSE (1.5%), LPS (1 μg/ml) or CpG (3 μM) for 16 h. The supernatant was collected and stored at −20 °C prior to cytokine quantification.

2.6. Quantification of CXCL-8 and cytokines assay

CXCL-8 concentrations in the cell supernatant were quantified using ELISA (BD Biosciences Pharmingen, Breda, The Netherlands) according to the manufacturer's instructions. The production of other inflammatory cytokines (TNF-α, IL-6, IL-1β and GM-CSF) was also measured in culture medium using Bio-Plex (Invitrogen) according to the manufacturer's instructions.

2.7. Preparation of whole cell extracts

HBE-14o cells were plated at a density of 5×10^6 cells/ml in 6-well cell culture plates and stimulated (as described earlier) for 6 h. Cells were washed twice with PBS and lysed with lysis buffer containing 20 mM Tris pH7.5, 1%Triton X-100, 100 mM NaCl, 40 mM NaF, and 1 mM EDTA with protease inhibitors (MiniTM protease inhibitors, Roche Diagnostics). Cells were subsequently lysed on ice for 5 min and following centrifugation at $3500\times g$ for 5 min, the supernatants (whole cell extracts) were collected and frozen at −70 °C.

2.8. Western blot analysis of inflammasome signaling

The protein concentration was determined by BCA protein assay kit (Pierce). The lysate (25 μg) was subjected to SDS/PAGE [10% (w/v) gel]. The separated proteins were electroblotted on PVDF membranes (Bio-Rad). Membranes were then washed once with Tris/HCl, pH 7.4, containing 159 mM NaCl and 1% Tween 20 (TBS-T), and then blocked in super-blocking buffer (Pierce) for 1 h. After washing the membranes with TBS-T, antibodies against IL-1β and caspase-1 were added for 24 h at 4 °C as indicated by the supplier. After three washes with TBST, membranes were treated for 1 h with HRP-conjugated goat anti-rabbit IgG for IL-1β and rabbit anti-goat IgG for Icaspase-1 diluted to 1:20,000 in TBS-T. After three washes with TBS-T, immunoreactive protein bands were revealed with an enhanced chemiluminescence Western blot analysis system (Amersham Pharmacia Biotech). Films were scanned and analyzed on a GS7-10 Calibrated Imaging Densitometer equipped with Quantity One v. 4.0.3 software (Bio-Rad).

For detection of equal loaded protein on the gel, the membranes were stripped with stripping buffer (Pierce), incubated with antibody to GAPDH as a housekeeping protein and visualized by ECL.

2.9. FACS analysis

Cells (1×10^6/ml) were incubated with CSE (1.5%) or CpGODN (3 μM), and LPS (1 μg/ml) for 2 h, then stained with phycoerythrin (PE)-conjugated anti-human TLR9 Ab (eB72-1665, ebioscience) or a PE-Rat IgG2a class-matched irrelevant Ab (eBioscience) as control, PE-TLR4 Ab (12–9917, ebioscience) or PE-mouse IgG2a for 30 min in immunofluorescence buffer (PBS 1%, FBS 1% and sodium azide 0.1%) on ice. Then cells were washed three times with immunofluorescence buffer and 10,000 cells were analyzed on a FACSCanto flow cytometer (Becton Dickinson, USA). The results obtained with specific antibodies were compared with those using isotype-matched control antibodies in parallel. For intracellular TLR9 staining, cells were fixed in fixation buffer (Becton Dickinson) for 10 min and then, after washing with permeabilizing buffer (Becton Dickinson), incubated for 30 min with TLR9 antibody in permeabilizing buffer.

2.10. Cell viability assay

Viability of cells was determined by staining cells with Annexin-V or 7-ADD by flow cytometry analysis (FACS) as described before [42].

2.11. Statistical analysis

All conditions were performed in triplicate, and all experiments were repeated up to five times. Results are presented as mean ± S.E.M. Data from two conditions, such as between stimulated and control samples, were compared using the unpaired 2-tailed, student's *t*-test. Analyses were performed using GraphPad Prism (version 2.01). Results were considered statistically significant when $p<0.05$.

3. Results

3.1. CSE induces cytokine production by HBE-14o cells

CSE induced the release of CXCL-8 from HBE-14o cells in a concentration-dependent manner (Fig. 1A and B). 3% CSE slightly affected the viability of the cells, an effect which was not observed with 1.5% (data not shown). Therefore, 1.5% (OD 0.06) was used in all subsequent experiments. CSE exposure also induced the release of significant amounts of IL-1β, GM-CSF and IL-6 (Fig. 1C, D, and E) but not IL-10 (Fig. 1F) and TNF-α (data not shown).

In addition, the effects of LPS (TLR4), CpGODN (TLR9) and MSU (inflammasome activators) on cytokine release were examined. Stimulation of HBE-14o cells with LPS, CpGODN and MSU resulted in the release of CXCL-8 and IL-1β, as well as IL-6 (Fig. 1B, C and E) but not IL-10 (Fig. 1F). CpGODN, like CSE, induced a significant release of GM-CSF (Fig. 1D).

3.2. CXCL-8 production by CSE is mediated by inflammasome and TLRs signaling

To explore whether CSE regulates CXCL-8 production via inflammasome signaling we incubated cells with AZ11645373 (100 nM) or Z-VAD-DCB (10 μM). Preincubation of the HBE-14o cells with AZ11645373 or Z-VAD-DCB inhibited the release of CSE-induced CXCL-8 production (Fig. 2). These results are indicative for involvement of inflammasome signaling in production of CXCL-8.

Next, the involvement of ROS and NO in the production of CXCL-8, cells was studied. As shown in Fig. 2 preincubation of cells with L-NAME and NAC abrogated the release of CXCL-8 induced by CSE.

In previous studies we demonstrated that CSE exposure of human macrophages and human neutrophils can activate TLR4 and TLR9 resulting in the release of CXCL-8 [19,26,41]. Next the involvement of these receptors in CSE-induced CXCL-8 production from HBE-14o cells was investigated. Pretreatment of HBE-14o cells with anti-human TLR4 neutralizing antibody partially blocked CXCL-8 secretion in response to CSE (Fig. 3), while no inhibition was observed when the cells were preincubated with mouse IgG2a isotype control (Fig. 3). To investigate the involvement of TLR9, cells were preincubated with inhibitory ODN without a CpGODN motif which resulted in a suppression of the CSE-induced CXCL-8 production by HBE-14o cells (Fig. 3). LPS and CpGODN served as positive controls in these experiments (Fig. 3).

3.3. Cigarette smoke modulates inflammasome signaling by stimulation of caspase-1 and maturation of IL-1β expression

To determine whether the CSE components are capable of inducing caspase-1 activation, cells were stimulated with either CSE, CpGODN or MSU or the positive control LPS. Cell lysates were then prepared and analyzed for caspase-1 activation and IL-1β expression. CSE induced an up-regulation of the expression of caspase-1 and IL-1β (17 KD) in HBE-14o cells (Fig. 4A and B). Similar results were obtained with CpGODN, LPS and MSU (Fig. 4A and B).

3.4. HBE-14o cell regulation of TLR4 and 9 expression via CSE

The expression of TLR4 and TLR9 was determined on whole and permeabilized HBE-14o cells to determine the location (cell surface or intracellular) of these receptors (Fig. 5). HBE-14o cells did express TLR4 on their cell surface membrane which was significantly decreased after CSE exposure (Fig. 5A). Similar results were obtained after LPS exposure but not after CpGODN exposure (Fig. 5A). It is generally accepted that TLR9 is expressed inside cells. However, intestinal epithelial cells have been shown to express surface TLR9 on their surface [12]. In the present study we demonstrated that human

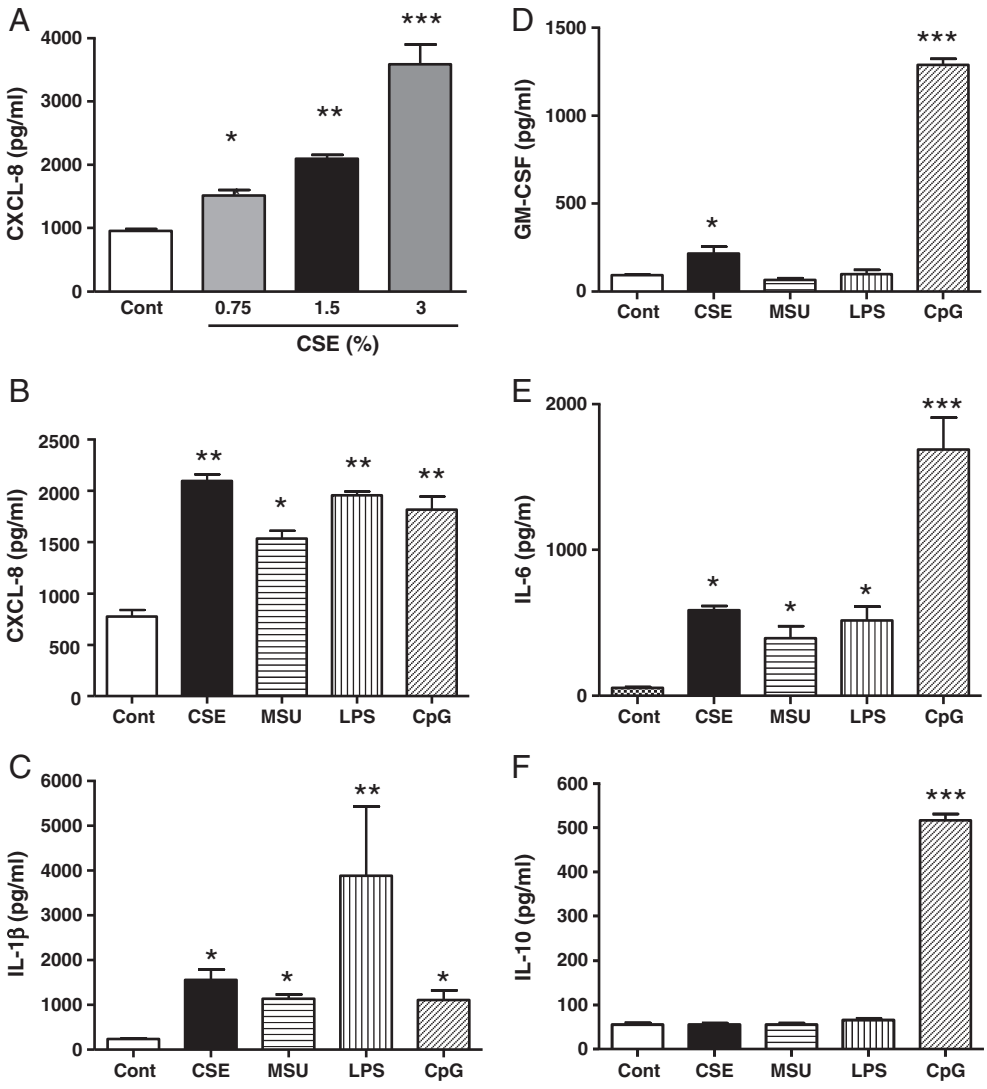

Fig. 1. Effects of CSE on cytokine release of HBE-14o cells. HBE-14o cells (10^6/ml) were seeded onto 96-well plates and placed in low-serum (1% FBS) medium and stimulated with various concentrations of (A) CSE or (B–F) CSE (1,5%), MSU (1 μg/ml), LPS (1 μg/ml), CpGODN (3 μM) for 16 h. Levels of CXCL-8 (A, B), IL-1β (C), GM-CSF (D) IL-6 (E) and IL-10 (F) in supernatants were measured by using a ELISA (for CXCL-8) or 20-plex kit (Invitrogen/Biosource) and values are expressed as pg/ml. Assays were performed in duplicate three times. Values are expressed as mean +/− S.E.M. (n = 3). *p ≤ 0.05 significantly different compared with control, **p ≤ 0.01 significantly different compared with control, ***p ≤ 0.001 significantly different compared to control.

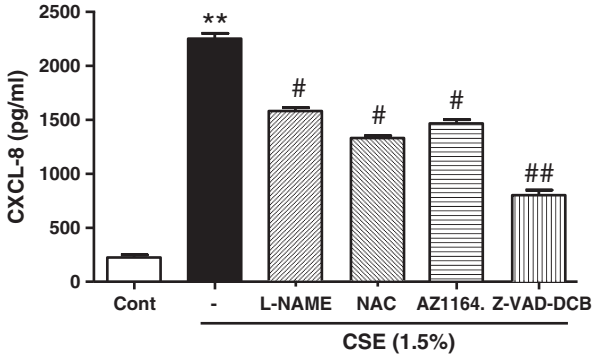

Fig. 2. Effects of pharmacologic inhibitors for release of CXCL-8 by HBE-14o cells. HBE-14o cells (10^6/ml) were seeded onto 96-well plates and placed in low-serum (1% FBS) medium and were left untreated or pretreated with L-NAME(10 nM) or NAC (10 nM), AZ11645373 (100 nM) and ZVAD-DCB (1 μM) for 30 min and then stimulated with CSE (1.5%) for 16 h. Levels of CXCL-8 in supernatants were measured by ELISA and values are expressed as mean +/− S.E.M. (n = 3). **p ≤ 0.01, significantly different compared to control and # P ≤ 0.05, ## P ≤ 0.01 significantly different compared to cells treated with CSE alone.

bronchial epithelial cells also express TLR9 on their surface (Fig. 5C). We also found that CSE significantly decreased the surface expression of TLR9, an effect which was not observed with LPS or CpGODN (Fig. 5C). The down-regulation of TLR4 expression on the surface of epithelial cells by CSE and LPS could be explained by internalization. Indeed, the TLR4 expression was increased in permeabilized cells (Fig. 5B). In contrast, such an effect was not observed for TLR9 (Fig. 5D).

4. Discussion

Full understanding of the molecular mechanisms underlying the development of COPD remains incomplete, but in vitro studies using human airway epithelial cells may contribute to furthering our understanding of this disease. Toxic substances in cigarette smoke are able to activate diverse signal transduction pathways and induce inflammatory mediators in the lungs. Thus, exploring each compartment of the respiratory system individually will help unravel each step of the patho-physiological processes behind lung emphysema. Hence, studying airway epithelial cells, that maintain mucosal integrity and

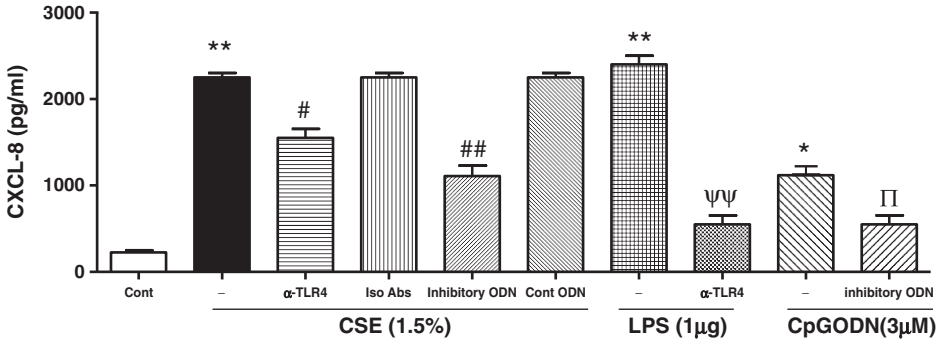

Fig. 3. Effects of blocking TLR4 and TLR9 on CXCL-8 release induced by CSE. HBE-14o cells (10^6/ml) were seeded onto 96-well plates and placed in low-serum (1% FBS) medium and were left untreated or pretreated with naturalizing antibodies against TLR4 (20 μg/ml) or isotype control IgG, inhibitory ODN without CpGODN motif (25 mM) or control ODN (5 mM) for 30 min and then activated with CSE (1.5%), LPS (1 μg/ml) or CpGODN (3 μM) for 16 h. Levels of CXCL-8 in culture supernatants were measured by ELISA as described in Material and methods. Assays were performed in duplicate three times. Values are expressed as mean +/− S.E.M. (n = 3). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ significantly different compared to control, $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$ significantly different compared to CSE and $^{\Psi\ \Psi}p \leq 0.01$ significantly different to LPS and $^{\Pi}p \leq 0.05$ significantly different compared to CpGODN.

induce/control pulmonary inflammation [3,8], could render valuable information.

COPD is a chronic disease which is driven by neutrophilic inflammation. Amongst other cytokines, it has been well recognized that one of the most prominent chemokines in COPD is CXCL-8. The levels of this chemokine are increased in sputum from COPD patients and correlate with the increased number of neutrophils found in the lungs [14,17]. More recently, IL-1β has received special attention, since this cytokine is involved in the prolonged and sustained inflammatory responses. The role of IL-6 in the pathogenesis of lung emphysema and COPD is suggested by studies showing that high amounts of serum or sputum IL-6 are related to impaired lung function or a faster decline in lung function in COPD patients [10,49]. Here, we demonstrated that CSE induced the production of IL-6 by HBE-14o cells. This response was not seen with human neutrophils [26], macrophages [40] and dendritic cells [29] and may therefore be specific to epithelial cells.

CXCL-8 and IL-1β can be released via the stimulation of TLRs. Previously, we demonstrated that cigarette smoke releases chemokines and cytokines from human inflammatory cells via TLR signaling. In this study now, we aimed to explore the response of human bronchial epithelial cells to cigarette smoke and studied the interaction of TLRs and inflammasome signaling. We have demonstrated that CSE induces the production of CXCL-8, IL-1β and IL-6 from human bronchial epithelial cells. Comparable results were found with TLR4 and TLR9 ligands and an inflammasome activator. Inflammasome/caspase-1 signaling is important for the maturation and production of IL-1β [38]. Moreover, it was demonstrated that IL-1β is involved in the production of CXCL-8 by bronchial epithelial cells via direct activation or by prolonging the half-life of CXCL-8 mRNA [11,32]. CSE also increased the production of IL-1β and GM-CSF. Although, CXCL-8 was increased as well, this does not indicate a causal relationship.

It is generally accepted that DAMPs and PAMPs induce IL-1β expression in human monocytes [36]. We found that CSE induces 1) the release of IL-1β and 2) the release of CXCL-8 via TLR4, 9 and inflammasome activation from HBE cells. Thus, we can draw the conclusion that airway epithelial cells play a critical role in initiation and maintaining of lung disorders induced by cigarette smoke. The release of CXCL-8 after CSE incubation of human bronchial epithelial cells [25] and HBE-14o cells [31] has been described previously.

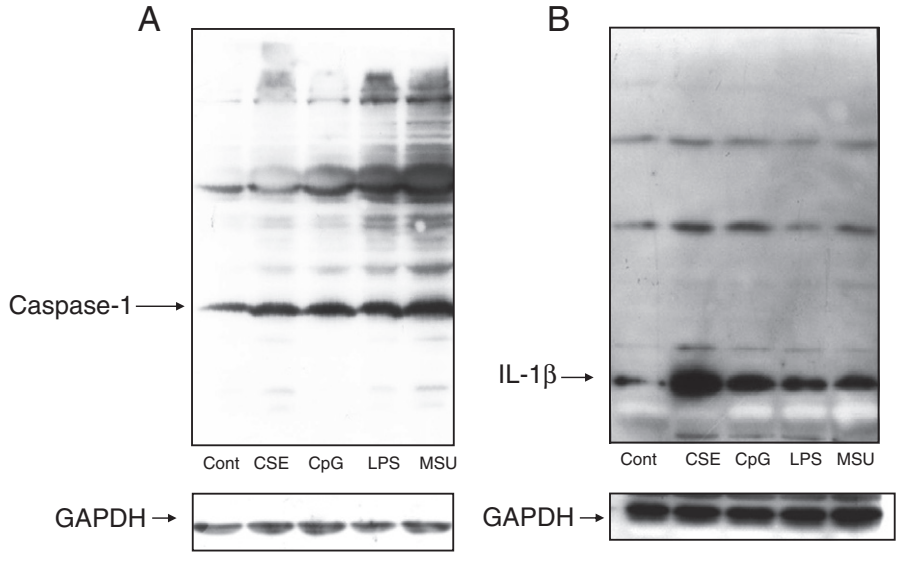

Fig. 4. Modulation of caspase-1 and IL-1β in HBE-14o cells. Immunoblots of whole cell extracts obtained from HBE-140 (5×10^6) stimulated with CSE (1.5%), LPS (1 μg/ml), CpGODN (3 μM) or MSU (1 μg/ml) for 6 h. 25 μg of cell lyzates were subjected to SDS-PAGE gels (10%) and blotting, for the expression of caspase-1 (A) and IL-1β (B) as described in material and methods. Immunoblots are representative of at least three independent experiments. After stripping the blots, the membranes were incubated with GAPDH antibody (Stressgen) as a housekeeping protein and visualized by ECL.

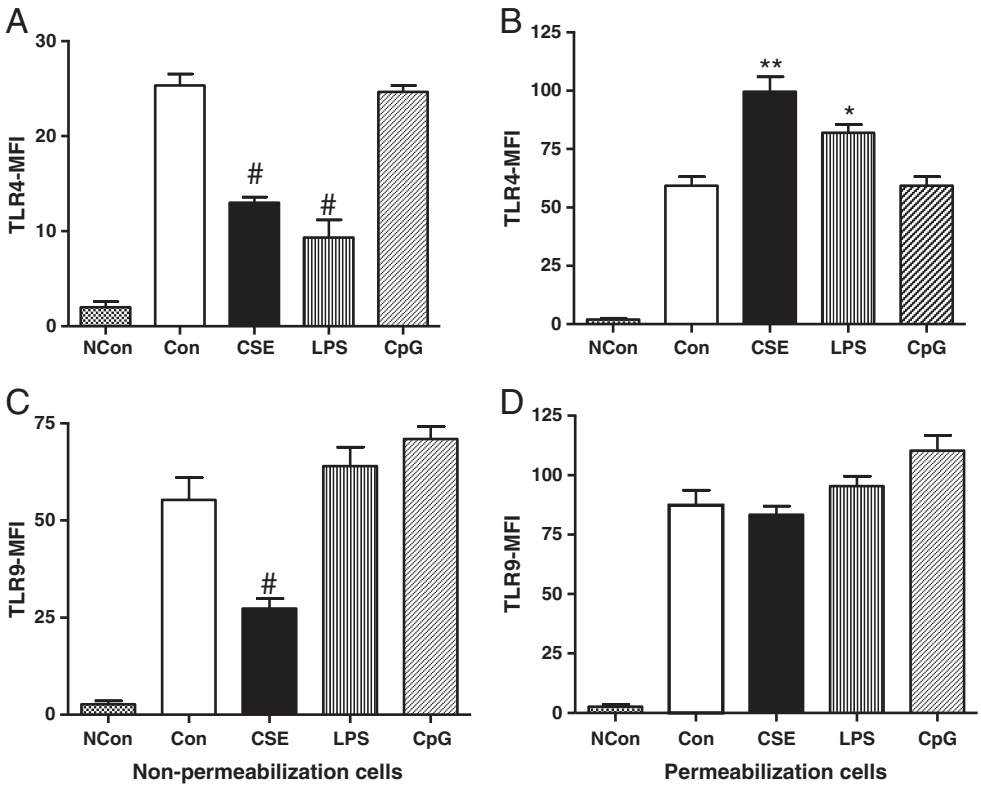

Fig. 5. Flow cytometric analysis of TLR4 and TLR9. HBE-14o cells were treated with CSE (1.5%), LPS (1 μg) or CpG (3 μM) for 2 h and the surface and intracellular expression of TLR4 and TLR9 were determined by staining the cells by PE-conjugated antibodies against TLR4, TLR9 or isotype control antibodies as described in Materials and methods. FACS analysis of a representative of at least 3 experiments showing the mean fluorescence intensity (MFI) difference of each group. Values are expressed as mean +/− S.E.M. *$p \leq 0.05$, **$p \leq 0.01$ significantly enhanced compared to control; # $p \leq 0.05$ significantly reduced compared to control (stained cells). The bars indicate the means and the error bars indicate the standard deviations of triplicate measurements obtained in three separate experiments. NCon: non treated control (unstained cells).

However, a blocking peptide against TLR4 did not suppress the release of CXCL-8 [31]. In contrast, using human inflammatory cells and TLR transfected cells, we demonstrated that TLR4 and TLR9 are involved in CSE induced cytokine production [19,26]. Moreover, we showed that the release of CXCL-8 induced by CSE was suppressed when TLR4 neutralising antibodies were applied. There could be a discrepancy between the use of an antibody against TLR4 and the TLR4 blocking peptides [31]. Besides, regulatory effects by CSE of TLR4 expression on human epithelial cells (up-regulation by increasing the production) and macrophages (down-regulation of surface and up-regulation of intracellular levels) has been reported [31,41]. In addition, we have shown that CSE decreased the surface expression of TLR4 on HBE-14o cells. This could be due to internalization of the receptors after CSE exposure, an explanation supported by the higher expression of TLR4 in permeabilized cells.

TLR9 recognizes bacterial and viral DNA, and studies using synthetic single-stranded ODNs defined the immunogenic sequences of bacterial DNA recognized, consists of unmethylated CpG motifs in the context of species-dependent surrounding sequences [21,24]. TLR9 is expressed primarily in antigen presenting cells such as B cells and dendritic cells. Several lines of evidence suggest that the molecular recognition of CpGDNA occurs inside the cell, perhaps in lysosomes. In contrast to other TLRs, TLR9 has not been detected on the surface of cells and TLR9 and CpGDNA localize to a common intracellular compartment [16]. Ewaschuk et al. were the first to describe a surface expression of TLR9 on intestinal epithelial cells [12], and now we have also demonstrated that TLR9 is expressed on the surface of human bronchial epithelial cells. It can be speculated that TLR9 is expressed on the outer membrane due to the strategic position of intestinal and airway epithelial cells in relation to microbes. TLR9 surface expression is decreased after CSE stimulation, an effect which was not observed with LPS or CpGODN. Unlike TLR4, the decrease in TLR9 surface expression could not be explained by internalization since there was no difference in TLR9 expression between control and CSE exposed permeabilized cells.

We then wanted to investigate the molecular pathways leading to CXCL-8 production after TLR stimulation by CSE. In previous studies it was demonstrated that reactive oxygen species and the purinergic P2X7 receptor were involved in CSE-induced CXCL-8 release by human neutrophils [26,27]. Indeed, ROS pathway inhibitors (L-NAME and NAC) and the purinergic receptor antagonist AZ1164 partly prevented CSE-induced CXCL-8 production, indicative of the involvement of these pathways in the production of CXCL-8 by CSE. This was further confirmed by using the caspase-1 inhibitor ZVAD-DCB, which reduced the CXCL-8 production by 70%. In addition, the protein expression of caspase-1 was increased in HBE-14o cells after CSE stimulation.

P2X7 is a receptor for ATP and impacts on many signal transduction pathways, including the inflammasome [9]. We found that the release of CXCL-8 was suppressed by a P2X7 receptor antagonist. Earlier studies demonstrated that extracellular ATP is a strong IL-1β-releasing agent for macrophages [7]. Evidence based on in vitro and in vivo studies with $P2X7R^{-/-}$ mice identified the receptor responsible for ATP-dependent IL-1β release as the P2X7 receptor [13,22,45].

The most pronounced expression of IL-1β was observed with CSE (as compared with CpGODN and MSU) which might be explained by the fact that CSE stimulates both TLR4 and TLR9 and contains more products that may interfere with cell signaling. Generation of reactive oxygen species is considered a crucial element for NLRP3 (inflammasome signaling) activation [47]. Moreover, activation of NALP3 leads to release of IL-1β from cells, thus it can be suggested that CSE by induction of ROS, activation of TLR4 and TLR9 and inflammasome signaling induces CXCL-8 production.

CXCL-8 and IL-1β are considered as important mediators in the pathogenesis of COPD [34]. Reduction in IL-1β also resulting in reduced CXCL-8 levels may suppress the influx and activation of neutrophils thereby breaking the sustained inflammatory condition in COPD. It is well recognized that tobacco, due to fermentation, is contaminated with microbes from which the cell wall components (LPS), DNA or RNA can activate TLRs [33,39]. In conclusion, human bronchial epithelial cells incubated with cigarette smoke results in the release of IL-1β and the expression of caspase-1 which is indicative for inflammasome activation.

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