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Cigarette smoke induces CXCL8 production by human neutrophils via activation of TLR9 receptor

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Abbreviations

BSA; Bovine serum albumin

cDNA; complementary DNA

CSE; Cigarette smoke extracts

FACS; Fluorescence activated cell sorter

HRP; Horse radish peroxidase

NF-κB; Nuclear factor κB

NAC; N-acetyl- cysteine

IRAK; IL-1R-associated kinase;

LPS; Lipopolysaccharide

L-NAME; N^G-monomethyl L-arginine

NF-kB; Nuclear Factor kappa B

ODN; Oligonucleotide;

PMN; Polymorphonuclear cells

PMA; Phorbol myristate acetate

PE; Phycoerythrin

MFI; Mean fluorescence intensity

SDS-PAGE; Sodium dodecyl sulfate–polyacrylamide gel electrophores

SEAP; Secreted embryonic alkaline phosphatase

TLR; Toll like receptor

TRAF6; TNFR-associated factor 6

Abstract

Chronic obstructive pulmonary disease (COPD) is a major health problem and cigarette smoke is the main risk factor for the development of COPD. The characteristic changes in airway morphology, inflammatory cell infiltration and mediator expression in COPD may result from direct effects of cigarette smoke on airway cells. Toll-like receptors (TLRs) are key elements in pathogen recognition by the host immune system. Although TLRs have been intensely studied in innate immunity and infection their critical role in non-infectious challenges has only recently emerged.

Here we investigate whether cigarette smoke induces TLR9- signaling in human neutrophils. Human neutrophils were isolated from buffy coat and exposed to cigarette smoke extract. The production of CXCL8 was measured as a functional readout and the role of TLR9 signaling was investigated. Cigarette smoke extract induced CXCL8 release via TLR9 activation in neutrophils, which was confirmed in TLR9 stably transfected HEK293 cells. Moreover, cigarette smoke extract upregulated the expression of TLR9 and the upregulated expression was suppressed by N-acetyl cysteine

TLR9 mediates cigarette smoke-induced release of CXCL8 and this may contribute to the accumulation of neutrophils and inflammation within the airways of smokers.

Introduction

Chronic obstructive pulmonary disease (COPD) is recognized as a major cause of death worldwide and posses an increasing global healthcare problem [1]. As previously stated [2] the definition of COPD recognizes the 'abnormal', exaggerated or amplified inflammatory response in the lung and systemically to cigarette smoking. The pattern of inflammation recruitment of lymphocytes, macrophages and neutrophils as well as activation and damage to structural cells following the release of inflammatory chemokines and cytokines [2-5]. In the Western world the major driver of disease is cigarette smoke which is a complex mixture of organics, heavy metals and reactive oxygen species (ROS) [6-11]. Importantly, Sopor [12] highlighted that chronic inhalation of cigarette smoke can modulates both innate and adaptive immune responses. Moreover, it has been speculated that many of the health consequences of chronic inhalation of cigarette smoke might be due to its adverse effects on the immune system [13].

Smokers likely develop airway inflammation through oxidative stress. Cigarette smoke activates macrophages and neutrophils to releases proinflammatory mediators, chemokines and elastolytic enzymes [14]. CXCL8 is an important chemokine produced by macrophages, neutrophils and epithelial cells and induces the recruitment of neutrophils to the airways [15,16]. As a part of the innate immune response, pattern recognition receptors mediate the interaction between conserved patterns on microorganisms and the host. Toll like receptors (TLR) are pathogen-associated molecular pattern receptors for diverse microbial-derived molecules expressed predominantly on innate immune cells [17]. To date, eleven TLR family members have been identified in the human genome [18,19]. Bacterial DNA containing unmethylated CpG motifs act as important regulators of human neutrophil

functions via TLR9. For example, stimulation of the TLR9 pathway via CpG oligonuucleotides (ODN), induces CXCL8 production by neutrophils via the generation of peroxynitrite (ONOO-) [20].

Recently, we and others have demonstrated that cigarette smoke extract (CSE) activates TLR4 signaling [17, 21, 22]. Furthermore, TLR4 seems to plays a critical role in the development lung emphysema [23]. In the current study we investigated whether CSE could also regulate TLR9 signaling in neutrophils. Thus, the present study was conducted to clarify TLR9 activation and expression in human primary neutrophils in response to CSE using the production of CXCL8 as a functional readout for TLR9 activation. Moreover, the involvement of reactive oxygen species and a major signal transduction pathway (Nuclear Factor κB, NF-κB) were investigated by the use of pharmacological inhibitors. The activation of NF-κB by CSE via TLR9 activation was also determined using genetic methods by overexpression of TLR9 in receptor-deficient cells and the subsequent analysis of NF-κB-reporter gene activity.

Material and Methods

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 calf serum, nonessential amino acids, DCFH-DA (D399) and diaminofluorescein diacetate (DAF) were purchased from GibCo BRL Life Technologies (GIBCO-BRL-Invitrogen Corporation, Carlsbad, CA, USA). Rabbit polyclonal antibody against TLR9 was obtained from Santa Cruz Biotechnology (Tebu-bio, Heerhugowaard, The Netherlands). 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). CpG ODN 2395 stimulatory oligonuleotide, 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', Blasticidin S and QUANTI-Blue™ reagent were purchased from Invivogen (Cayla-InvivoGen Europe, Toulouse, France). Anti-TNFR-associated factor 6 (TRAF6) (catalog no. sc-7221) and anti-IRAK-1 Ab (catalog no. 06-872) antibodies were purchased from Santa Cruz Biotechnology and Upstate (Haarlerbergweg, The Netherlands), respectively. Bicinchoninic acid (BCA) protein assay kit and super-blocking buffer were purchased from Pierce (Perbio Science B.V, Etten-Leur, The Netherlands).

Protein A-Sepharose bead slurry and ECL or ECL Plus were purchased from Amersham Biosciences (Buckinghamshire, UK). TRIzol reagent and p-nifty 2xNF-κB

SEAP plasmid and Sybergreen Universal PCR Master Mix and SuperScript III reverse transcriptase were purchased from Invitrogen (Carlsbad, California, USA). Trans-AM NF-κB p65/NF-κB p50 Transcription Factor Assay Kit (Active Motif, Rixensart, Belgium). PE- labeled TLR9 and isotype IgG control were purchased from ebioscience (San Diego, CA, USA).

Preparation of cigarette smoke extracts (CSE)

Cigarette smoke-conditioned medium (CSM) was produced as previously described before [17]. CSE was generated by the burning of commercially available Lucky Strike cigarettes without filter (British–American Tobacco, Groningen, The Netherlands) with tar 12 mg, nicotine 0.9 mg and CO 9 mg 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). Briefly, this machine was used to direct main- and side stream smoke from one cigarette through a 5-ml culture medium (RPMI without phenol red). Hereafter, absorbance was measured spectrophotometrically and the media was standardized to a standard curve of CS medium concentration against absorbance at 320 nm and this number was taken as 100%. The pH of the resultant extract was titrated to pH 7.4 with medium to different concentrations (0.75-3%). A 1.5% solution was used in the present study following preliminary experiments, which indicated that this was non-toxic (viability ≥ 96%) and demonstrated a good induction of CXCL8 release [24].

Isolation of neutrophils

Human neutrophils were isolated from healthy volunteers as previously described

[25]. Briefly, human neutrophils were obtained from heparinized venous blood buffy coat by Ficoll-Hypaque centrifugation, followed by sedimentation in 5% dextran/0.9% Saline. Neutrophils were separated from erythrocytes by lysis in a solution of 0.15 M NH4C1, 0.01 M NaHCO and 0.01 M tetra EDTA. The recovered neutrophils were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplied with 10% fetal calf serum and essential amino acides and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2 and washed three times. The purity of neutrophils preparations was >93-95%, as determined by Wright's staining of cytospin preparations. Cell viability of these cells was 97%, as determined by trypan blue exclusion. Neutrophils, as isolated above, were kept on ice until used as described below. The **n** number for each experiment represents the number of separate individual donors used for each individual study.

Cell lines

TLR9 stably transfected HEK 293 cell lines (293XL-hTLR9, Catalog no 293xl-htlr9 for TLR9) and null HEK293 (293-null, Catalog no. 293-null) were purchased from Invivogen. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% FCS and 10 mg/ml of Blasticidin S (an antibiotic) each 3 days.

Cell activation

Neutrophils (5 x 10^6 cells/ml) were incubated for 20–30 min with L-NAME (0.1-10 mM) or NAC (0.1-10 mM); chloroquine (1-50 mg/ml) (enhances endosomal pH and thereby blocks lysomal pathways and used here as a blocker of TLRs signaling)[26] or blocking anti-sense ODN (1-10 mM) and then stimulated with CSE (1.5%), CpG ODN type A (3 μM) or control ODN (5 mM) for 30 min for Western blot analysis. For

CXCL8 determination $3x10^6$cells/ml were incubated for 9 h according to the conditions for Western blot analysis. For determination of intracellular CXCL8 expression, HEK293 stably transfected TLR9 or null cells were stimulated with CSE or CpGODN for 6 h, and the whole cells extracts were subjected for Western blot analysis. For preparation of samples for RT-qPCR, cells were activated for different time points with CSE or CpG ODN as described in below. The viability of cells before and after each experiments were determined by staining with propidium ionide (PI) or 7-ADD labeled with FITC by using flow cytometery (FACS analysis).

Quantification of CXCL8 and cytokine assay

CXCL8 concentrations in cell supernatants 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) was also measured in culture medium using CBA Kits (BD Biosciences Pharmingen) and flow cytometry (FACScalibur) according to the manufacturer's instructions.

Measurement of intracellular ROS stress and NO

Cells ($1x10^6$/ml) were activated for with CSE (1.5%) or CpG ODN (3 μM) for 1 and 5 h. After 2 washes with cold PBS, cells were preincubated with the 10 μM redox-sensitive dye DCFH-DA (D399) for measurement of ROS and 5 μM of Diaminofluorescein diacetate (DAF) for determination of NO for 20 min as previously described [22,23]. Intra cellular levels of ROS and NO were determined by flow cytometry (FACScalibur). The data were plotted and analyzed using CellQuest software.

Anti-TLR neutralization of cytokine production

Cells were incubated with anti-human TLR2 (clone TL2.1) or mouse IgG2a isotype control (20 μg/ml), for 30 min at room temperature or with anti-human TLR4 (clone HTA125) or mouse IgG2a isotype control (20 μg/ml), (all from eBioscience, CA, USA) for one hr at 37°C. Hereafter, cells were stimulated with CSE (1.5%) and incubated overnight. Supernatants were collected and stored at -20°C prior to CXCL8 quantifications.

Preparation of whole cell extracts

Neutrophils were plated at a density of 5×10^6 cells/ml in 6-well cell culture plates and stimulated (as described above) for 30 min. Cells were washed twice with PBS and lysed with lysis buffer containing 20mM Tris pH7.5, 1%Triton X-100, 100 mM NaCl, 40 mM NaF, 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 xg for 5 min, the supernatants (whole cell extracts) were collected and frozen at -70 °C.

Preparation of cytoplasmic and nuclear extracts

Cells after activation were washed twice with PBS and allowed to equilibrate for 5 min in ice-cold cytoplasmic extraction reagent (Pierce) containing protease inhibitors (MiniTM protease inhibitors, cocktail). Following centrifugation at 3500 xg for 5 min, the supernatants (cytoplasmic extracts) were collected and frozen at -70 °C. The pellets were re-suspended in nuclear extraction buffer (Pierce) containing protease inhibitors. After vigorous mixing and incubating for 10 min on ice, the solution was centrifuged at 14,000 xg for 5 min, and the supernatant (nuclear extract) was

collected and stored at -70 °C. Protein concentrations were determined by using a bicinchoninic acid (BCA) protein assay kit.

Immunoprecipitation of endogenous IRAK-1 and Western blotting

Neutrophils ($15x10^6$) were stimulated with CSE (1.5%), CpG ODN (3 μM) for 10 min or pretreated with inhibitory ODN (10 mM) for 30 min and then stimulated with CSE (1.5%) for 10 min. After the incubation for the indicated times, reactions were stopped with 5 ml of ice-cold saline with 2 mM phenylmethylsulfonylfluoride (PMSF) and rapid centrifugation. Then, the pellets were immediately frozen in dry ice after aspiration of the supernatants. Afterward, the pellets were lysed with 0.5 ml of ice-cold extraction buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 40 mM-glycerophosphate, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 2 mM DTT, 20 mM NaF, 2 mM sodium orthovanadate, 5 mM PMSF, 100 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin. Following a 15-min incubation period on ice, samples were briefly vortexed, transferred to microtubes, and centrifuged at 13,000 x g, for 10 min, at 4°C. Supernatants were collected, and protein concentration was determined using the Micro BCA Protein Assay Reagent kit, according to the manufacturer's instructions (Pierce). Extracts with equal amounts of proteins were used for immunoprecipitation. The cell lysates (500 μg) were precleared by mixing with control rabbit IgG Abs (matched isotype) plus protein A-Sepharose beads for 1 h at 4°C. Then, 4 μg of rabbit polyclonal IgG specific for IRAK-1 was added to the precleared lysates and incubated at 4°C for 2 h on a rotator. Then, immune complexes were captured by the addition of 50 μl of prewashed protein A-Sepharose bead slurry and by incubation in a rotator for 1 h at 4°C. Sepharose beads were washed three times in ice-cold lysis buffer and then mixed 1:1 with 2x sample buffer

and boiled 5 min. Then, samples were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane and immunoblotted with rabbit polyclonal Ab anti-TRAF6 and detected with ECL.

Western Blotting

For Western blot analysis, cells were washed once with cold PBS and lysed in ice-cold lysis buffer containing 50 mM Tris (pH 8.0), 110 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 100 µg/ml PMSF. Protein concentration was determined by BCA protein assay kit and (30-50 mg) protein were subjected were subjected to SDS/PAGE [10% (w/v)] gel. The separated proteins were electro- blotted on PVDF membranes. Membranes were then washed once with Tris/HCI, pH 7.4, containing 159 mM NaCI and 1% Tween 20 (TBS-T), and then blocked in super-blocking buffer for 1 h. After washing with TBS-T, the membranes were probed with antibodies against TLR9 and IRAK-1 and diluted of 1:3000 in TBS-T and incubated for overnight. After three time washes with TBS-T, membranes were treated for 1 h with HRP-conjugated indicated antibodies 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 (ECL) or ECL Plus. Films were scanned and analyzed on a GS7-10 Calibrated Imaging Densitometer equipped with Quantity One v. 4.0.3 software.

Quantification of NF-κB activity

NF-κB activation was detected using the Trans-AM NF-κB p65/NF-κB p50 Transcription Factor Assay Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. Briefly, 2 µg of the nuclear extracts was incubated with

an oligonucleotide containing the NF-κB consensus site bound to a 96-well plate. After extensive washes, the NF-κB complexes bound to the oligonucleotide were incubated with an antibody directed against the NF-κB p65 subunit at a dilution 1:1,000. After washes, the plates were subsequently incubated with a secondary antibody conjugated to horseradish peroxidase (1:1,000), and the peroxidase reaction was quantified at 450nm with a reference wavelength of 655 nm.

FACS analysis

Isolated neutrophils were incubated with CSE (1.5%) or CpGODN (3 μM) alone or in combination at various time points and then fixed with formaldehyde (1%) and then permeabilized with permeabilization buffer (eBioscience) and stained with phycoerythrin (PE)-conjugated anti-human TLR9 Ab (eB72-1665) or a PE conjugated Rat IgG_2a class-matched irrelevant Ab (eBioscience) as control for 30 min in permeabilization buffer on ice. PMN, defined as $CD16^+CD3^-CD19^-HLA\text{-}DR^-$ cells (all antibodiies by ebioscience), were gated at a purity of >95.2%.

Cells were washed three times with immunofluorescence buffer (PBS 1%, FCS 1% and sodium azide 0.1%) and 10,000 cells were analyzed on a FACSCalibur flow cytometer. The results obtained with specific antibodies were compared with those using isotype-matched control antibodies in parallel.

RNA isolation and Real-time PCR

TotalRNA for cDNA synthesis was prepared from activated and unactivated control cells at different time points and then isolated using the TRIzol reagent, and reverse transcription was performed in a 20-μl reaction with 1 μg total RNA, 50 mM Tris-HCl (pH 8), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM each deoxynucleotide

triphosphates and SuperScript III reverse transcriptase at 42 °C for 1 h. To eliminate DNA contamination, the RNA samples were incubated with DNase I at room temperature for 15 min. For real-time PCR, cDNA was analyzed for the expression of TLR9 and b2-microglobulin (B2M) genes using Sybergreen Universal PCR Master Mix by using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) under conditions of 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The sequences for PCR primers were: CXCL8: Forward 5'- CTGGCCGTGGCTCTCTTG-3'; Reverse 5'-CCTTGGCAAAACTGCACCTT-3', (Accession number: NM_000584), TLR9: Forward 5'-TGGTGTTGAAGGACAGTTCTCTC-3' and Reverse 5'-CACTCGGAGGTTTCCCAGC-3' (Accession number: NM_017442) and B2M: Forward 5'-CTCCGTGGCCTTAGCTGTG-3' and Reverse 5'-TTTGGAGTACGCTGGATAGCCT-3' (Accession number: AF072097).

Stable transfection of NF-κB plasmid and NF-κB activity assay

TLR9 stably transfected HEK 293 cells were maintained in DMEM containing 10% fetal calf serum in 5% CO2 at 37°C. Cells were split on a 6-well dish or 60 mm dish at density 0.75×10^5cells per dish. After 18 h, the cells were transfected with p-nifty 2xNF-kB SEAP plasmid using Lipofectamine Plus reagent according to the manufacturer's instructions. The total amount of DNA was adjusted using an empty vector, pcDNA3 (Invivogen). Cells were washed and cultured with 1mg/ml Zoesin for 4 weeks. At week 5 of transfection, cells were activated with CpG ODN (3 μM) and or CSE (1.5%) for 9 h for detection of SEAP by supernatant. For determination of SEAP as an indicator for NF-kB activity, the supernatants were subjected to QUANTI-Blue™ reagent as instructed by the manufacturer. The amount of SEAP

levels was determined spectrophotometrically at 620-655 nm.

Statistical analysis

Experimental results are expressed as mean ± S.E.M. Results were tested statistically by an unpaired two-tailed Student's t-test or one-way ANOVA, followed by Newman–Keuls test for comparing all pairs of groups. Analyses were performed by using GraphPad Prism (version 2.01). Results were considered statistically significant when $p < 0.05$.

Results

CSE and CpG-induces CXCL8 release via the generation of NO and ROS stress

CpGODN induced the production of CXCL8 release by neutrophils in a concentration-dependent manner (Fig. 1A). In all subsequent experiments a sub-maximal concentration of 3 μM CpG ODN was selected. CSE (1.5%) also induced CXCL8 release from human neutrophils (Fig. 1B and C). This effect on CXCL8 release was selective as CSE did not significantly induce the release cytokines such as TNF-α and IL-6 and chemokines such as CCL2, CXCL10 and CCL11 (data not shown). Pre-incubation of the neutrophils with L-NAME or NAC (inhibitors of NO or ROS generation, respectively) inhibited the release of both CSE- and CpG ODN (3 μM)-induced CXCL8 production back to baseline (Figs 1B & 1C). Pre-incubation of cells with the combination of sub-maximal concentrations of NAC and L-NAME further suppressed the CXCL8 production (Fig. 1D) compared to that seen with either treatment alone although the levels did not return to baseline. Furthermore, CSE (1.5%, Fig. 1E) and CpG ODN (3μM, Fig. 1F) enhanced intracellular ROS and NO production (*$p \leq 0.05$, **$p \leq 0.01$).

CSE activates TLR9 signaling

To explore whether CSE regulates CXCL8 production via TLR9, neutrophils were pre-treated with chloroquine, an inhibitor of TLRs, particularly TLR9, activation pathways. Chloroquine (1-50 mg/ml) concentration-dependently attenuated the release of CXCL8 induced by CSE (Fig. 2A upper panel). Chloroquine was cytotoxic from concentrations ≥10 mg/ml (Fig. 2A, lower panel). Next, to examine specific activation of TLR9 signaling by CSE, the effects of the TLR9 inhibitory ODN on CXCL8 release was investigated [24]. Inhibitory ODN suppressed the CSE-induced

CXCL8 production in a concentration-dependent manner (Fig. 2B). We then determined the effects of neutralizing TLR2 and 4 antibodies on CXCL8 release by human neutrophils. As shown in Fig. 2C, a neutralizing antibody against TLR4 significantly attenuated the release of CXCL8 by human neutrophils. A combination of CQ and the neutralizing antibody against TLR4 further suppressed the release of CSE-induced CXCL8 production (Fig.2C).

Further evidence for TLR9 involvement was suggested by the effects of CSE on TLR9 in stably transfected HEK293 cell line. CSE induced the release of CXCL8 in TLR9 stably transfected HEK293 cells but not in TLR9 null cells (Fig. 3A and B). CpGODN served as a positive control for activation of TLR9 pathways (Fig. 3). Components of cigarette smoke such as nicotine and acrolein may also modulate TLR9 signaling but we were unable to demonstrate any production of CXCL8 by these compounds in TLR9 stably transfected HEK293 cells (data not shown).

CSE modulates TLR9 expression in neutrophils

TLR9 is predominantly expressed in intracellular vesicles [17]. Neutrophils were first fixed and permeabilized, and subsequently stained with a PE-conjugated anti-TLR9 Ab or isotype IgG control. After short exposure (5 h) to CSE, the intracellular expression of TLR9 was upregulated (Fig. 4A) whereas after overnight exposure (24h) TLR9 expression was downregulated (Fig. 4B). Moreover, CSE did not induce surface expression of TLR9 (data not shown). This regulation of TLR9 protein expression paralleled with expression of mRNA (Fig. 5A). Similar results were observed with CXCL8 mRNA expression (Fig. 5B). Both NAC (1mM) and L-NAME (0.1μM) inhibited CSE and CpGODN-enhanced intracellular TLR9 protein expression (Fig. 5C).

CSE induces activation of NF-κB via TLR9 signaling

In human neutrophils, CSE increased the activity of NF-κB in the nucleus and pretreatment with NAC, L-NAME and the TLR9 inhibitory ODN suppressed NF-κB activity induced by CSE (Fig. 6A).

TLR activation triggers a signaling cascade that involves sequential recruitment and activation of IRAK-4 and -1 [30]. Phosphorylation of IRAK-1, in turn, enables recruitment and phosphorylation of the TAK1 complex which then dissociates, along with TRAF6, from the receptor complex. TRAF6 and IRAK-1 are ubiquitinated and degraded enabling induction of TAK1 kinase activity and activation of NF-κB and the up-, regulation of proinflammatory gene transcription [30].

We, therefore, investigated whether CSE or CpGODN affected IRAK-1 expression and the association of IRAK-1 with TRAF6. IRAK-1 underwent partial degradation upon stimulation with CSE and CpG ODN as evidenced in immunoblots of whole-cell extracts (Fig. 6B). Pre-incubation of CSE-stimulated cells with the blocking inhibitory ODN abrogated IRAK-1 degradation. IRAK-1 was associated with TRAF6 in resting neutrophils (Fig. 6C, lane 1). However, this association was lost following incubation of cells with CpG ODN and CSE for 10 min (Fig. 6C, lanes 2 and 3, respectively) in agreement with the notion that IRAK becomes dissociated and degraded. The inhibitory ODN abrogated the dissociation and degradation of IRAK-1 (Fig. 6C, compare lanes 3 and 4).

Next, as supportive evidence for CSE actions on the TLR9 pathway, the regulation of NF-κB in stably transfected dual TLR9/NF-κB reporter HEK293 cells was investigated. The production of SEAP (secreted embryonic alkaline phosphatase), an indicator of NF-κB activation in this system, was significantly enhanced after CSE

incubation (Fig. 7A). Similar results were obtained with CpGODN as a positive control. The response was specific for TLR9 signaling since SEAP production was not increased by CSE or CpG in HEK 293 lacking TLR9 (Fig. 7B).

Discussion

The present study demonstrates that CSE induces the production of CXCL8 from human neutrophils via TLR9 signaling through a ROS- and NO-dependent mechanism. Chloroquine and the TLR9 inhibitory ODN attenuated the release of CXCL8-induced by CSE. Moreover, inhibitory ODN abrogated the induction of CXCL8 mRNA by CSE and inhibitory ODN also blocked TRAF6 and IRAK1 association and degradation and subsequent induction of NF-κB activity induced by CSE. Due to the short span of neutrophils in culture and their low transfection efficiency, we were not able to modulate TLR9 expression or signaling pathways in these cells. In support of our hypothesis, we report that CSE was able to induce CXCL8 release in HEK293 cells stably transfected with TLR9 but not in cells devoid of TLR9. Internalization and endosomal maturation have been shown to be required for CpG DNA to activate TLR9 signaling in immune cells [31, 32]. Chloroquine, which effectively blocks endosomal maturation, significantly inhibited the CSE-induced increase of CXCL8 production in neutrophils, indicating a similar signaling pathway as CpGODN.

In COPD patients, an increase of proinflammatory cytokines and chemokines including TNF-α and CXCL8 has been reported, and these mediators play an important role in establishing and maintaining the inflammatory condition, characterized by high local neutrophilia [4]. Importantly, neutrophils constitutively express TLR9 [33-35]. TLRs have been intensely studied in the context of microbial challenges to inflammatory and immune cells, but their critical role in non-infectious challenges has only recently emerged. TLRs are the best characterized pattern recognition receptors in neutrophils, in which lies most of their pathogen recognition capacity [33]. Moreover, TLRs recognize distinct structural components of pathogens

and trigger a signaling cascade that involves association of its intracellular Toll-IL-1R (TIR)-signaling domain with the adaptor molecule MyD88 [36]. A sequential recruitment andactivation of IRAK-4 and 1 then occurs [37]. Phosphorylation of IRAK-1, in turn, enables recruitment and phosphorylation of the TAK1 complex which then dissociates, along with TRAF6, from the receptor complex. TRAF6 and IRAK-1 are ubiquitinated and degraded enabling induction of TAK1 kinase activity and activation of NF-κB and the up-regulation of proinflammatory gene transcription [30]. From this signaling complex, downstream cascades ultimately lead to activation of NF-κB, regulating proinflammatory gene transcription [30]. TLRs are expressed in numerous cells within the airway and can therefore act as important sensors of environmental particulates and gases [23, 38, 39]. TLRs have also been implicated in the pathogenesis and severity of autoimmune [40], cardiac and lung diseases [23,38]. In accordance with this concept, we and others [17,23, 41,42] have shown that cigarette smoke modulates TLR2 and 4 expression. In addition, CSE contains LPS [43] which is a potent activator of TLR4 signaling. Using a neutralizing antibodies against TLR4 and TLR2, we have shown a partial involvement of TLR4 in CSE-induced CXCL8 expression in primary human neutrophils. Moreover, blocking TLR4 activity in combination with attenuation of TLR9 using chloroquine resulted in a greater decrease in CSE-induced CXCL8 expression. This indicates a degree of cross-talk between TLRs in regulating the response to cigarette smoke. The expression and coupling of receptors in different cell types will affect various signaling pathways as well as that of NF-κB which may affect the final functional response observed. The fact that there is a residual level of CXCL8 production remaining after blocking TLR4 and TLR9 indicates the involvement of other pathways stimulated by CSE in the control of CXCL8 production.

Interestingly, we have found that CSE up-regulates TLR9 and CXCL8 expression in parallel. Moreover, the up-regulation of TLR9 expression was dependent on ROS and NO generation and reversed by longer term incubation. The down-regulation of receptor expression by CSE is probably not due to cell toxicity since the viability of the cells was not affected. Further investigations are required to understand these pathways and also the precise mechanisms that drive TLR activation.

TLR9, is to date the best characterized sensor for bacterial DNA, containing short sequences of unmethylated CpGODN motifs [38] and TLR9 stimulation results in alterations in cellular redox balance, peroxynitrite formation and activation NF-κB [44-49]. Taken together, it can be concluded that CSE plays a crucial role in the induction of TLR9 expression and activation through generation of ROS and NO and the subsequent release of CXCL8 in neutrophils. TLR4 is also implicated in this effect and it is likely that other TLRs may also modulate CXCL8 production by CSE in an analogous manner.

Accumulating data indicated that cigarette smoke induces the production of free radicals in cells [47, 48]. Free radicals in turn are able to activate a number of key pro-inflammatory transcription factors including NF-κB and AP-1 [49, 50] Indeed, CSE induced the cellular production of NO and ROS which could be protected by L-NAME and NAC. Inhibitory ODNs, that block TLR9 activation, attenuated CSE-induced NF-κB activity and upstream events in TLR9 signaling including the effects on TRAF and IRAK-1. Furthermore, CSE activated NF-κB in TLR9 stably transfected, but not untransfected, HEK293 cells.

The precise component(s) of CSE that stimulate TLR9 and the mechanism by which it acts remains to be elucidated. It is possible that CSE contains CpG ODN which can directly stimulate TLR9 [44, 46, 51]. However, the differential effect of CSE and

CpG ODN on ROS production suggest that this may not be the case. It has been suggested that ROS may activate TLRs either directly or indirectly [52, 53] potentially through an effect on modifying key residues within the TLR complex [54]. Furthermore, future studies are needed to determine whether the ROS is derived directly from CSE or as a result of CSE activation of neutrophils.

Several studies have demonstrated that activation of TLR9 by CpG ODN could prevent allergic airway inflammation and airway hypersensitivity reaction, suggesting a potential therapeutic application of TLR9 signaling in asthma [55]. In support of this, children who were exposed to parental smoking and those who took up cigarette smoking themselves, have a lower incidence of atopy to a range of common inhaled allergens [56]. Intriguingly, in the current study we show that CSE increases the expression and activity of TLR9 in a temporal manner which may contribute to the prevention of allergic reactions or the resolution of inflammatory responses.

The role of the neutrophil in COPD is complex and it is unclear what the predominant driving force for their activation is in the human airway. Here we report that CSE directly stimulates neutrophil functions but we cannot determine what is the initial event that occurs *in vivo?* It is possible that inhaled cigarette smoke activates neutrophils recruited into the airways as a result of CXCL8 production by epithelial cells for example or that cigarette smoke depositing on the airway epithelium could be absorbed and activate circulating neutrophils which then migrate to the airways. The use of animal models will be able to address this in the future.

The observations and data presented here suggest a link between TLR9 activation and the release of CXCL8 by human neutrophils upon CSE exposure (Fig. 8) which may contribute to the accumulation of neutrophils and inflammation within the

airways of smokers.

Author contribution:

E.M., designed, performed and wrote the paper. I.M.A and K.I. designed and analyzed the research data. A.K and F.N. edit the paper. G.F. was project leader and designed the experiments.

Acknowledgement:

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Figure legends:

Fig.1. Effects of L-NAME and N-acetyl-cysteine (NAC) on the release of CXCL8 and generation of reactive oxygen species (ROS) and NO induced by CSE and CpGODN of human neutrophils.

A) Neutrophils (10^6 /ml) were seeded onto 96-well plates and placed in low-serum (1% FCS) medium and stimulated with various concentration of CpGODN for 9 hours. Levels of CXCL8 in supernatants were measured by ELISA and values are expressed as pg/ml. Assays were performed in duplicate three times. Values are expressed as mean+/- S.E. (n = 3). ***p ≤ 0.001 significantly different compared to control.

Neutrophils (10^6 /ml) were seeded onto 96-well plates and placed in low-serum (1% FCS) medium and were left untreated or pretreated with various concentrations of L-NAME (B) or NAC (C) 30 min and then stimulated with CSE (1.5%) or CpG ODN (3 μM) for 9 hours. Levels of CXCL8 in supernatants were measured by ELISA and values are expressed as mean +/- S.E. (n = 3). ***p ≤ 0.001, significantly different compared to control (A) and **P ≤ 0.01, ***p ≤ 0.001 significantly different compared cells treated with L-NAME and NAC in activated cells with CSE and CpGODN (B and C).

D) Neutrophils were left untreated or pretreated with L-NAME (1 μM) or NAC (0.1mM) or in combination for 30 min and then stimulated with CSE (1.5%) or CpG ODN (3 μM) or control oligonucleotides (ODN control, 5 μM) for 9 hours. Levels of CXCL8 in supernatants were measured by ELISA and values expressed as mean +/- S.E. (n = 3). **p ≤ 0.01 significantly different compared to control, $^{\#\#}$ p ≤ 0.01 significantly different compared to CSE and ¶P ≤ 0.05 significantly different compared to CpGODN and $^{\$}$ p ≤ 0.05 significantly different compared to L-NAME alone or NAC

alone.

E and F) Neutrophils (10^6 cells) were incubated with CSE (1.5%) or CpGODN (3 μM) for 5 h. Cells were washed and preincubated with the 10 μM redox-sensitive dye DCFH-DA (for ROS) or 5 μM of Diaminofluorescein diacetate (for NO) for 20 min at 37 °C in PBS contains 1% BSA in dark place. Generation of intracellular ROS (E) and NO (F) were determined by FACS analysis as described in the methods and are representative of 3 independent experiments using PMN from different donors. The mean fluorescent intensity (MFI) of the groups are indicated in the figure. Analyses of MFI from 3 independent experiments are shown in the right hand side panels as mean±SEM.

Fig. 1

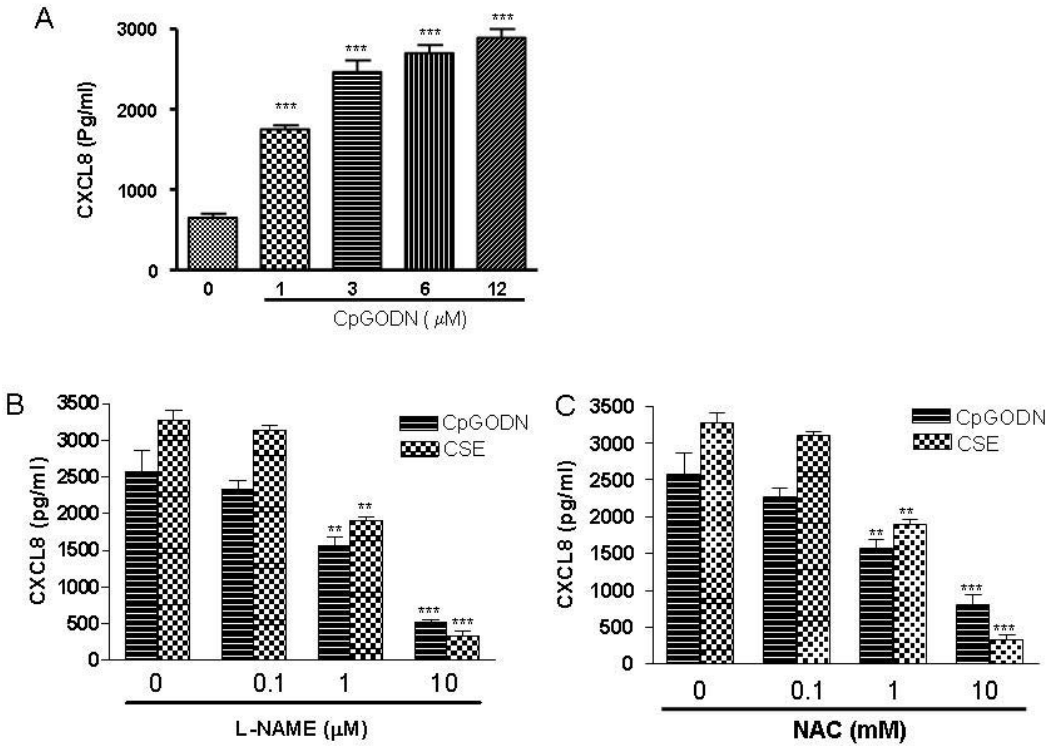
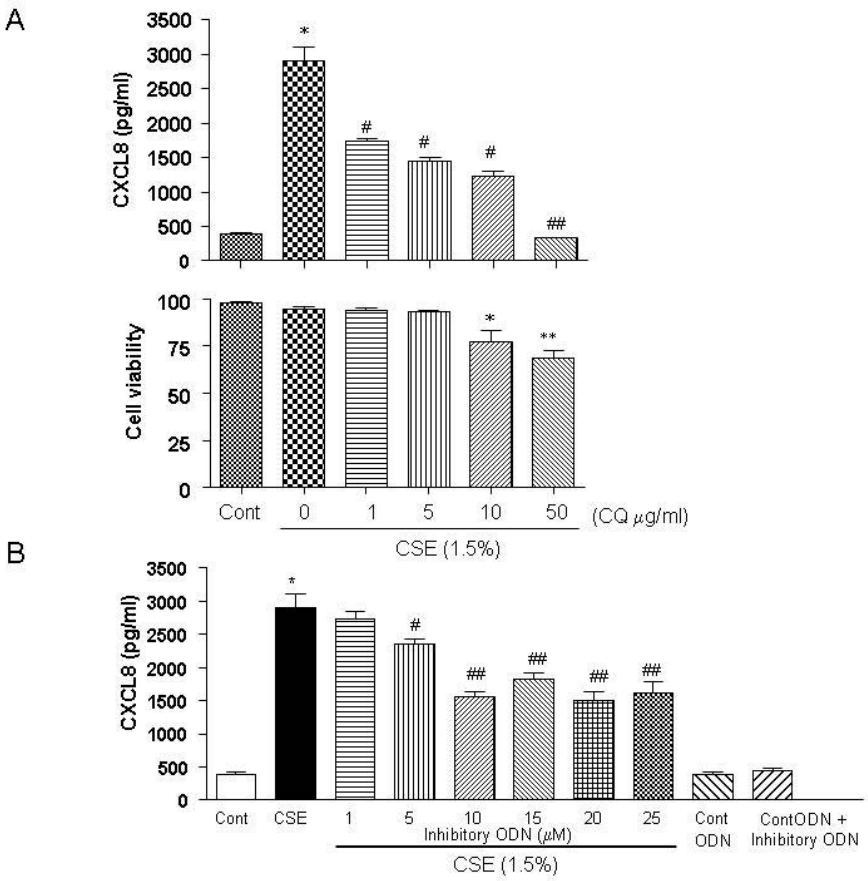


Fig. 2. Effects of chloroquine, TLR9 inhibitory ODN and blocking antibodies on CXCL8 release induced by CSE and CpGODN of human neutrophils.

Neutrophils (10^6 /ml) were seeded onto 96-well plates and placed in low-serum (1% FCS) medium and were left untreated or pretreated with chloroquine (CQ 1-50 µg/ml, A upper panel) or blocking inhibitory ODN (1 to 25 mM, B) or (C) naturalizing antibodies for TLR2 and TLR4 (20 µg/ml) with or without CQ for 30 min and then activated with CSE (1.5%) or control ODN (ODN Control 5mM) for 9 h. Levels of CXCL8 in culture supernatants were measured by ELISA and values are expressed as pg/ml. Assays were performed in duplicate three times. Viability of the cells after incubation with various concentrations of CQ was determined by staining of cells with PI (A lower panel). Values are expressed as mean +/- S.E. (n = 3). *P ≤ 0.05 significantly different compared to control, $^{\#}$ P ≤ 0.05, $^{\#\#}$ P ≤ 0.01 significantly different compared to CSE (A and B) and * P ≤ 0.05 significantly different compared to control, $^{\#}$ P ≤ 0.05 significantly different compared to CSE, $^{\$}$ P≤ 0.05 significantly different compared to CSE, ¶¶ P ≤ 0.01 significantly different compared to CSE (C). NO; Nitic oxide, CSE; cigarette smoke extract; CQ; Chloroquine.

Fig. 2


A
CXCL8 (pg/ml)
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3000
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Cell viability
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Cont
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1
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(CQ μg/ml)
CSE (1.5%)
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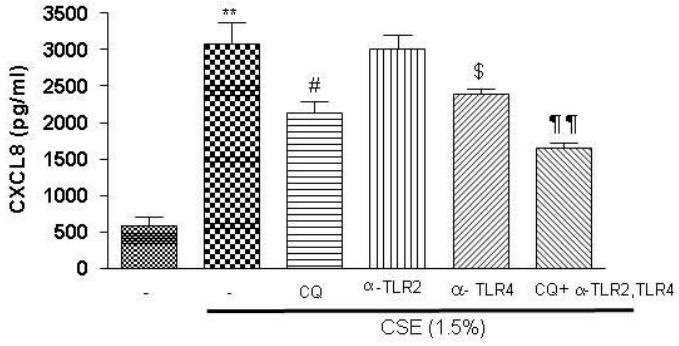
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CSE (1.5%)

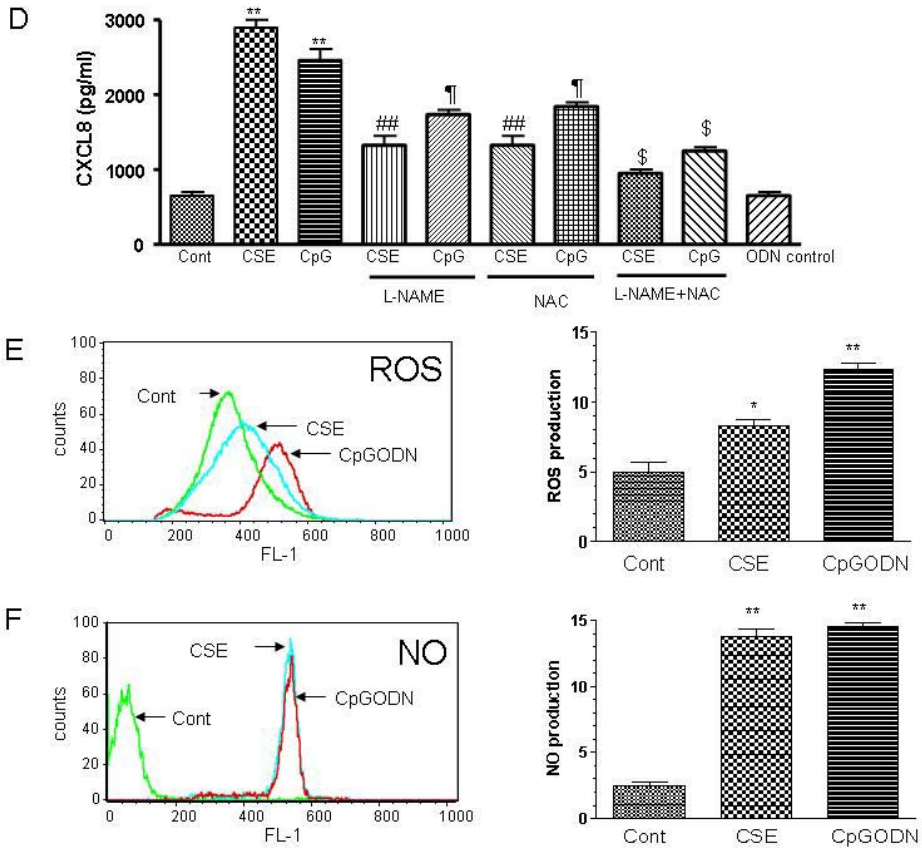


Fig.3. CSE induces the production of CXCL8 of TLR9 stably transfected HEK293 cell lines.

TLR9 stably transfected HEK293 and null HEK293 cells (2X10^6/ml) were stimulated with CSE (1.5%) and CpGODN (3 μM) for 9 h. CXCL8 levels in culture supernatants were measured by ELISA and values are expressed as pg/ml. Assays were performed in duplicate three times. Values are expressed as mean +/- S.E. (n = 3). **P ≤ 0.01, *** P ≤ 0.001 significantly different compared to control. (B) TLR9 stably transfected HEK 2943 or null cells stimulated for 6h with CSE or CpG ODN and after lysis whole extracts subjected to Western blot analysis with an anti-CXCL8 antibody or as a background with β-actin. The ratios of CXCL8 to β-actin expression from

three separate gels are shown in the lower panels. Data are mean±S.E.M. of triplicate samples. *p < 0.05 significantly different compared to control.

Fig. 3

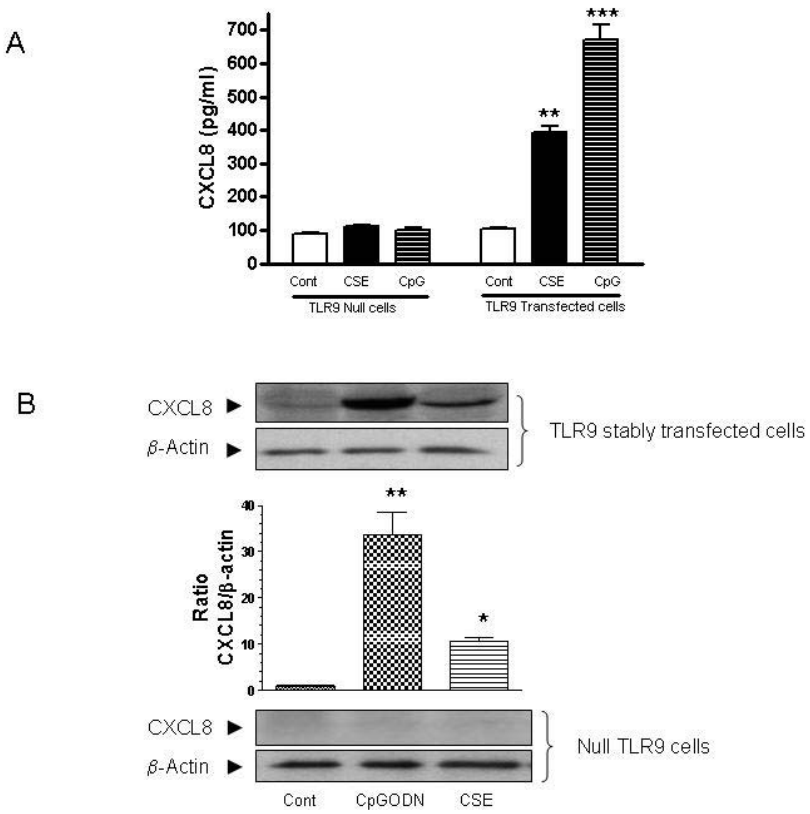


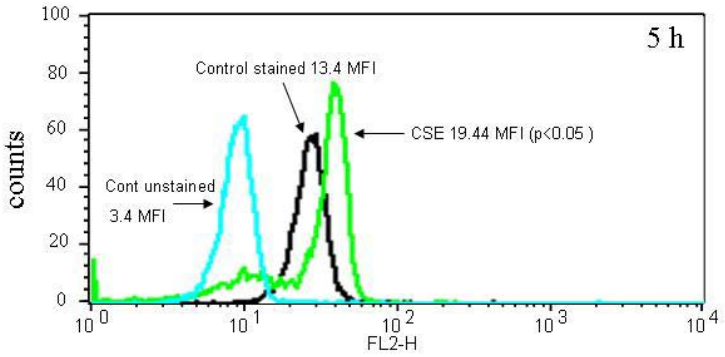
Fig. 4. CSE regulates TLR9 receptor expression.

Neutrophils (10^6/ml) were activated with CSE (1.5%) or CpGODN (3 μM) for 5 (A) and 24h (B). Then, cells were permeabilized and stained with an anti-human TLR9 antibody or an isotype control (IgG) for 30 min at dark place. After 2 washes with cold PBS the intracellular levels of TLR9 were determined by flow cytometry (FACS). The results are representative for four experiments using neutrophils from different donors. Blue lines indicated for control unstained cells, black lines indicated for control stained cells and green lines indicated for cells treated with CSE. The mean

fluorescent intensity (MFI) of the following groups are indicated in the figures.

Fig. 4

A



B

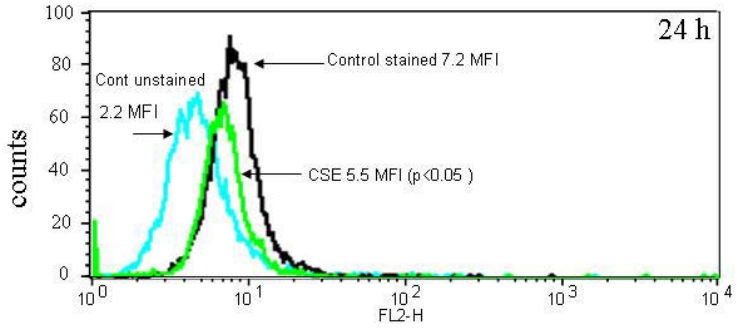


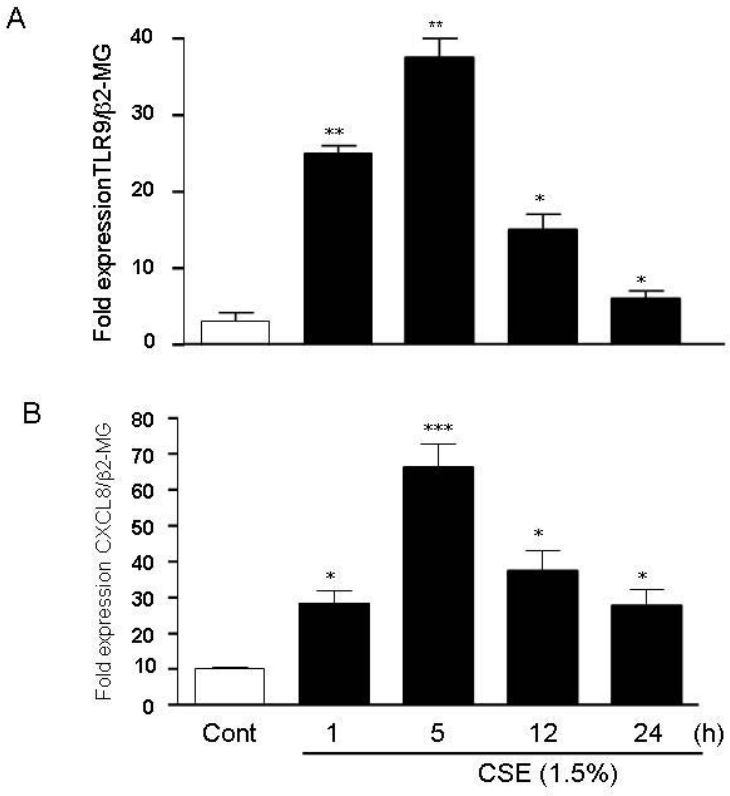
Fig.5. CSE modulates mRNA and intracellular expression levels of TLR9 and CXCL8 .

Neutrophils ($5x10^6$) were treated with CSE (1.5%) for 1, 5, 12 and 24 h. Total RNA were isolated and after conversion to cDNA was subjected to real-time PCR analysis for (A) mRNA expression for TLR9 or CXCL8 (B) as a ratio of β-Macroglobulin expression (A). Data are expressed as mean +/- S.E. and are obtained from three experiments. *p < 0.05, ** P ≤ 001 significantly different compared to control.

C) Neutrophils ($5X10^6$) were activated with CSE (1.5%) or CpGODN (3 μM) for 5h in the presence or absence of NAC (1 mM) or L-NAME (0.1μM) and then lyzed with lysis buffer. The expression of TLR9 and b-actin was detected by using 50 mg of

whole cell lysates by Western blot analysis. Blots are representative of 3 independent experiments showing similar results. The ratios of TLR9 to β-actin expression from three separate gels are shown in the lower panels. Data are mean ±S.E.M. of triplicate samples. *p < 0.05, ** P ≤ 001 significantly different compared to control and $^{\#\#}$p < 0.01, significantly different compared to CpG ODN and ¶¶p < 0.01, significantly different compared to CSE alone.

Fig. 5



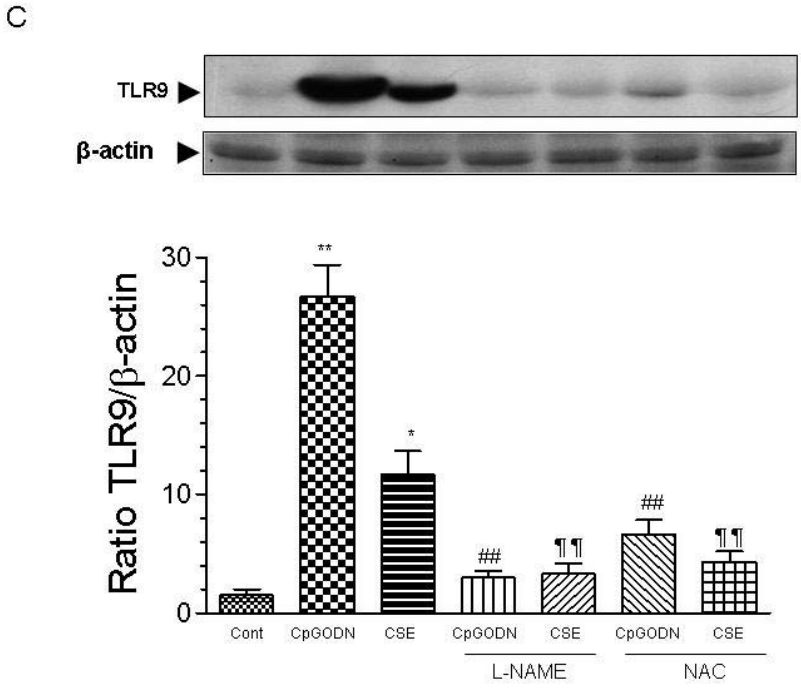


Fig.6. CSE activates TLRs and NF-κB pathways

Neutrophils ($5x10^6$) were pretreated with inhibitory ODN (10 μM) for 30 min and then stimulated with CSE (1.5%) or CpG ODN (3 μM). Nuclear extracts (10 μg) were subjected to EMSA reaction for detection of NF-κB activity as described on material and methods section (A). Values (mean ±SEM) are representative data from one of five independent sets of experiments. **p < 0.05, *** P ≤ 001 significantly different compared to control. # P ≤ 0.05 significantly different compared to CSE.

B) Immunoblots of whole cell extracts obtained from neutrophils ($15x10^6$) pretreated with inhibitory ODN (10μM) and then stimulated with CSE (1.5%) or CpGODN (3 μM) for 30 min revealed with a rabbit polyclonal anti-IRAK-1 Ab. Immunoblots are representative of at least three independent experiments. After stripping the blots,

the membranes were incubated with β-actin as a housekeeping protein and visualized by ECL. The ratios of IRAK-1 to β-actin expression from three separate gels are shown in the lower panels. Data are mean ±S.E.M. of triplicate samples. *p < 0.05 significantly different compared to control.

C) Neutrophils (10^6) were pretreated with inhibitory ODN and then stimulated with CSE or CpGODN for 10 min. Cells were lysed, and IRAK1 was immunoprecipitated from the supernatants using Abs specific for IRAK1. Proteins were resolved by SDS-PAGE and analyzed by Western blot (WB) with anti-phosphotyrosine monoclonal antibody (mAb) TRAF-6.

Fig. 6

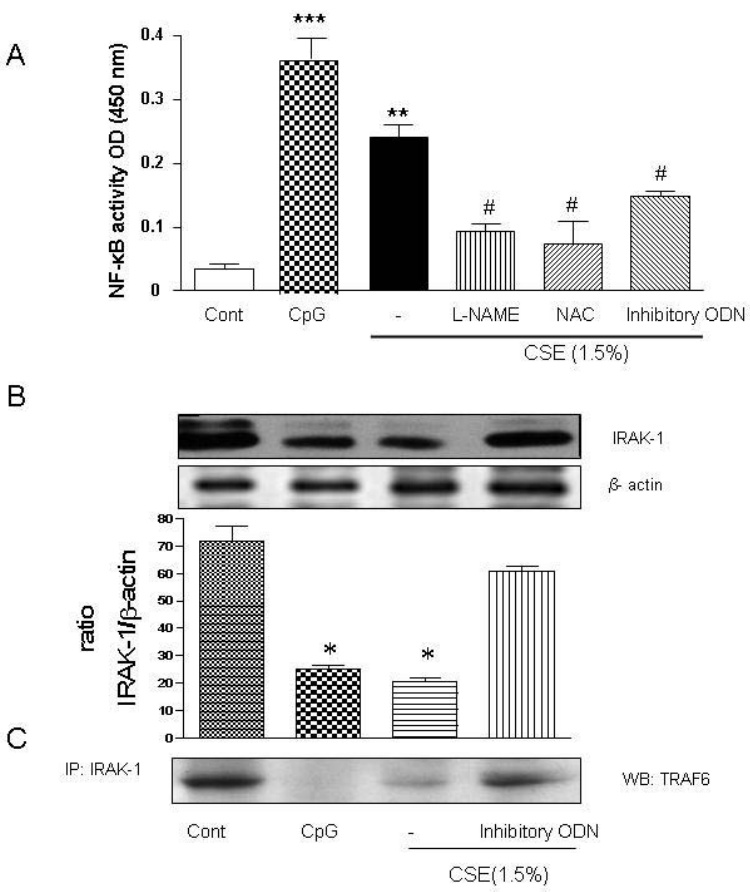


Fig. 7. CSE activates NFκB in TLR9 stably transfected HEK cells

TLR9, NFκB/SEAP double stably transfected HEK cells (A) and null, NFκB/SEAP

stably transfectd HEK cells (B) were activated with CSE (1.5%) or CpG ODN(3μM) for 30 min. Levels of SEAP as a indicator for NF-κB activity in supernatants were measured by QUANTI-Blue™ reagent. Assays were performed in duplicate three times. Values are expressed as mean +/- S.E. (n = 3). *p < 0.05 significantly different compared to control.

Fig. 7

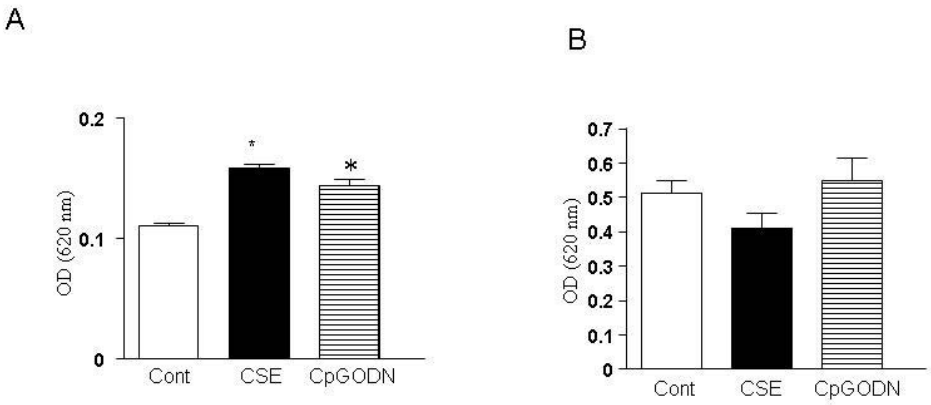
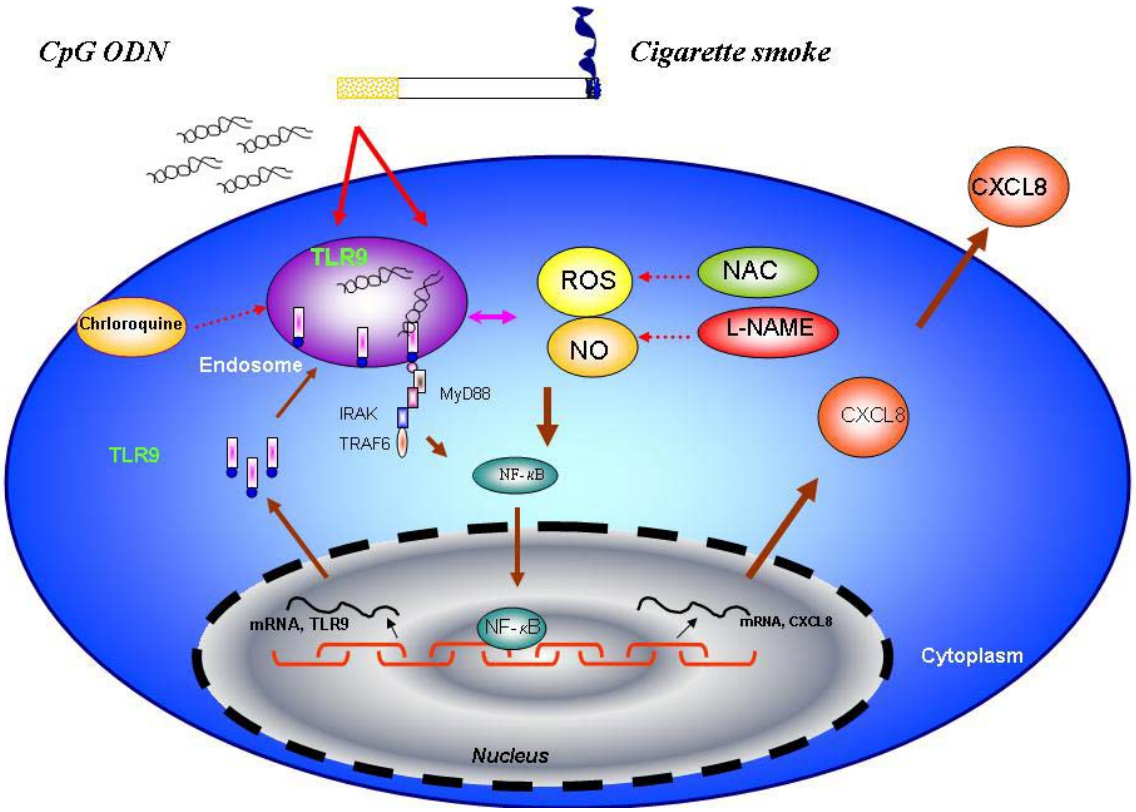


Fig. 8. Schematic diagram of the induction of CXCL8 by human neutrophils via activation of TLR9 receptors by cigarette smoke.

CSE and CpG ODN activate TLR9 pathway and generate ROS and NO production. These pathways may phosphorylates IRAK molecules. Following IRAK phosphorylation, the TRAF6 adaptor protein interacts and induces translocation of the transcription factor NF-κB to the nucleus, resulting in transcriptional activation of

genes encoding inflammatory mediators (e.g. CXCL8 and TLR9). NAC, L-NAME and chloroquine plays as blockers to the signaling.

Fig. 8



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