

ORIGINAL ARTICLE

Cigarette smoke suppresses *in vitro* allergic activation of mouse mast cellsE. Mortaz^{*,†,‡}, G. Folkerts^{*}, F. Engels^{*}, F. P. Nijkamp^{*} and F. A. Redegeld^{*}^{*}Division of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, 3508 TB Utrecht, The Netherlands, [†]Department of Biochemistry, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Iran and [‡]Department of Basic Science, Section of Biochemistry, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

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Summary

Background Mast cells are important effector cells in innate or acquired immunity that contribute to host defence. Excessive activation of mast cells can result in the development of allergic diseases, including atopic asthma. Mast cell activation by IgE and specific antigen induces the cells to release spasmogenic, vasoactive and pro-inflammatory mediators, which enhance airway smooth muscle contraction, vascular permeability and inflammatory cell recruitment. Recently, we have demonstrated that exposure of mast cells to cigarette smoke medium (CSM) triggered mast cells to produce chemokines. On the other hand, smoking may decrease the risk of allergic sensitization, which could be explained by a reduced IgE production or a diminished response of mast cells to activation of the IgE receptor.

Objective In this study, we investigated the effect of CSM on the allergic activation of mast cells through IgE and antigen.

Methods Primary cultured murine mast cells were exposed to CSM and activated with IgE and antigen or lipopolysaccharide (LPS). The release of granules, production of leukotrienes, chemokines and cytokines was determined in the supernatants by ELISA. The effect of CSM exposure on intracellular signalling, especially the nuclear factor (NF)- κ B and extracellular signal-regulated kinase (Erk)1/2 pathways, was analysed by Western blotting.

Results CSM suppressed IgE-mediated degranulation and cytokine release, but no effect was observed on leukotriene release. CSM induced phosphorylation of Erk1/2 in mast cells. In CSM-exposed mast cells, activating transcription factor (ATF)-1 was phosphorylated after stimulation with IgE/Ag. LPS-activated mast cells were not influenced by CSM.

Conclusion Our study suggests that exposure to cigarette smoke may lead to a reduced allergic activation of mast cells without affecting their response to activation via e.g. bacterial-derived LPS.

Keywords cigarette smoke, degranulation, IgE, IL-6, mast cell, TNF- α

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Introduction

Cigarette smoke (CS) has been considered a major player in the pathogenesis of chronic obstructive pulmonary disease (COPD) [1, 2]. COPD is a complex inflammatory disease that involves many different types of inflammatory and structural cells, all of which have the capacity to release multiple inflammatory mediators [3]. CS, directly or by inhalation of environmental tobacco smoke, might adversely affect the health of smokers and non-smokers [4], causing lung cancer [5], increased risk of respiratory infections, bacterial meningitis and the development of atopy and asthma during childhood [6, 7]. Despite these harmful effects, accumulating data suggest that tobacco

smoking, in addition to all its harmful effects, might protect smokers from some diseases. Epidemiological data indicate that smoking might decrease the incidence and/or the severity of several diseases such as Parkinson's disease, Sjögren's syndrome and Alzheimer's disease [8, 9]. It has been reported that mast cells and contents of their granules may have an impact on the pathogenesis of these diseases [10–13]. Moreover, chronic inhalation of CS alters a wide range of immunological functions, including innate and adaptive immune responses. It has been speculated that many of the health consequences of chronic inhalation of CS might be due to its adverse effects on the immune system [14]. Recently, it has been reported that parental smoking during childhood and

personal cigarette smoking in teenage and early adult life is associated with a lower risk of allergic sensitization in those with a family history of atopy. The underlying mechanisms for this association remain to be determined, but the findings are consistent with the hypothesis that the immune-suppressant effects of CS protect against atopy [15].

Exposure to CS activates an inflammatory cascade in the airway cells, resulting in the production of a number of potent cytokines and chemokines with accompanying damage to the lung epithelium, increased permeability and recruitment of macrophages and neutrophils to the airways [16]. Mast cells normally reside close to the epithelium, blood vessels, nerves and in the airways and gastrointestinal tract, near smooth muscle cells and mucus-producing glands [17]. Mast cells in these locations will be exposed to inhaled, or ingested, environmental challenges. Because mast cell activation results in the coordinated release of pro-inflammatory mediators into the surrounding tissue, inappropriate activation of this cell type following exposure to environmental challenges may result in a chronic inflammatory pathology [18–22]. Mast cells play a crucial role in acute and allergic inflammation, and have high-affinity receptors for IgE (FcεRI) on their surface [23]. Cross-linking of surface IgE molecules results in exocytosis of pre-formed mediators such as amines and proteases, as well as release of newly generated mediators including leukotrienes, prostaglandins and a variety of cytokines [24]. Protein tyrosine phosphorylation is an early and critical event in FcεRI-mediated signal transduction in basophils and mast cells [25]. After receptor aggregation, there is phosphorylation of the tyrosine residues in immunoreceptor tyrosine-based activation motifs of the β- and γ-chains, which then recruit the spleen tyrosine kinase (Syk)/ZAP-70 family protein tyrosine kinases and propagate downstream signals [26]. The phosphorylation of these downstream molecules then results in cell activation. The balance between the activity of protein tyrosine phosphatases and kinases regulates the extent of tyrosine phosphorylation and therefore the signal transduction that results in degranulation and the release of inflammatory mediators [27].

Extracts from CS bubbled through water have been used extensively to show that it can directly activate cells to promote pro-inflammatory effects. Recently, it has been demonstrated that tobacco-derived materials induce overproduction and secretion of proteinases [25] and attenuate degranulation via the release of nitric oxide (NO) [26]. We found that exposure to cigarette smoke medium (CSM) directly induces chemokine CCL3 and CXCL2 release from primary cultured mouse mast cells [27]. Therefore, CS may have dual effects on mast cells, increasing chemokine release, which induce trafficking of neutrophils into the lungs, and, on the other hand, CS suppresses the degranu-

lation, which is protective in allergic disorders. In this study, we explored the effect of CSM on the activation of murine mast cells. We show that CSM has dual effects on allergic activation of mast cells. CSM greatly reduced IgE-induced mast cell degranulation and production of cytokines TNF-α and IL-6. CSM did not suppress IgE-mediated leukotriene and chemokine production, nor did it affect activation by lipopolysaccharide (LPS).

Materials and methods

Reagents

Recombinant mouse IL-3 and Stem Cell Factor (SCF) were purchased from Peprotech (Sanvertech, Heerhugowaard, the Netherlands). LPS (*Escherichia coli* 055:B5), penicillin, streptomycin, 1-glutamine, sodium pyruvate, 2-mercaptoethanol and dinitrophenyl-conjugated human serum albumin (DNP-HSA) were obtained from Sigma-Aldrich (Sigma-Aldrich, Zwijndrecht, the Netherlands). RPMI 1640, Tyrode's buffer, fetal calf serum (FCS) and non-essential amino acids were purchased from GIBCO BRL Life Technologies (Invitrogen Corporation, Breda, the Netherlands). The ELISA kits for mouse TNF-α, IL-6, CCL3 and CXCL2 were purchased from Biosource (Invitrogen). Rabbit polyclonal antibodies against IκB-α and Syk, p65 were obtained from Santa Cruz Biotechnology (Tebu-bio, Heerhugowaard, the Netherlands). Mouse monoclonal antibody against phospho-CRE binding protein (CREB) (phosphorylated ATF-1 was also recognized by the antibody used to detect phosphorylated CREB), mouse monoclonal antibody against CREB, rabbit polyclonal antibody against phospho Erk1/2, and Erk1/2 were obtained from cell signalling (BIOKE B.V., Leiden, the Netherlands). The precision protein standards were purchased from Bio-Rad (Bio-Rad Laboratories, Veenendaal, the Netherlands). Horseradish peroxidase-conjugated rabbit-anti mouse IgG and goat anti-rabbit IgG were purchased from Dako (Dako Diagnostics, Heverlee, Belgium). Phosphotyrosine clone PY-20 (catalogue no. 610000) antibody was purchased from Becton Dickinson (BD Biosciences, Alphen aan den Rijn, the Netherlands).

Mouse bone marrow cultures

Bone marrow-derived mast cells (BMMC) were generated from the bone marrow of male BALB/cBy mice as described before [28]. Briefly, mice were killed, and intact femurs were removed. A sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe. The suspension of bone marrow cells was centrifuged at 320 × g for 10 min and cultured at a concentration of 0.5 × 10⁶ nucleated cells/mL in RPMI 1640 with 10% FCS (Sigma-Aldrich, Zwijndrecht, the Netherlands), 100 units/mL penicillin, 100 µg/mL

streptomycin (Life Technology), 10 µg/mL gentamycin, 2 mM L-glutamine and 0.1 mM non-essential amino acids (referred to as enriched medium) and a combination of IL-3 (5 ng/mL) and SCF (50 ng/mL) for 3 weeks at 37 °C in a humidified atmosphere with 5% CO₂. Non-adherent cells were transferred to fresh medium at least once a week. After 3–4 weeks, when a mast cell purity of >95% was achieved, as assessed by toluidine blue staining, the cells were used for the experiments.

Production of cigarette smoke medium

CS-conditioned medium (CSM) was produced following the method described before [29]. Briefly, a smoking machine (Teague Enterprises, Davis, CA, USA) was used to direct main and side stream smoke from one cigarette through 5 mL culture medium (RPMI without phenol red). Hereafter, absorbance was measured spectrophotometrically and the media were standardized to a standard curve of CS medium concentration against absorbance at 320 nm. The pH of the resultant extract was titrated to pH 7.4, and diluted with medium. This solution is considered to be 100% CSE. Solutions ranging from 0.75% to 1.5% were used in the present study in response to preliminary experiments that indicated that these were non-toxic concentrations. Non-toxic concentrations of CSM were detected performing different toxicological assays lactate dehydrogenase (LDH) and Fluorescence activated cell sorter FACS analysis (annexin-V and 7-AAD staining).

Activation of BMMC

In all experiments, unless specified otherwise, cells were cultured at a density of 1×10^6 cells/mL in 10 mL tubes containing 2 mL of complete medium at 37 °C in a humidified atmosphere with 5% CO₂. BMMC were incubated with CSM (1.5%). For activation of cells with IgE/Ag, cells were sensitized with 10 µg/mL monoclonal anti-dinitrophenyl IgE for 60 min at 37 °C. After washing twice with the culture medium, cells were stimulated with DNP-HSA (100 ng/mL). In some experiments, for activation of cells with LPS, cells were stimulated with 100 ng/mL for various time-points. Subsequent incubation times were chosen for optimal effects, i.e. 30 min for IκB-α degradation, phosphorylation of Erk1/2 and CREB, 5 h for TNF-α and IL-6 production and 16 h for CCL3 and CXCL2 release. Incubations were stopped by centrifugation of samples at $1200 \times g$ for 5 min at 4 °C. Cell viability was not affected by CSM (1.5%) ($93 \pm 1\%$ vs. $97 \pm 1\%$ in control) after 16 h.

FACS analysis of FcεRI

Mast cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgE receptor Ab (FcεRI) (BD Pharmingen) for 30 min at 4 °C, washed with PBS and

analysed on a FACS calibur flow cytometer (BD Biosciences). Rat IgG1, κ conjugated with FITC, was used as an isotype control antibody.

LTC4 release and mast cell degranulation assay

For LTC4 measurements, BMMC were incubated with CSM (1.5%) and sensitized with 10 µg/mL of anti-DNP IgE for 1 h, followed by stimulation with 10 ng/mL DNP-HSA. Sample supernatants were collected after 30 min and stored at -70 °C until analysis. LTC4 production was determined using an EIA kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. To assay degranulation, BMMC were pre-treated with NAC (10 mM) (30 min, 37 °C) and then activated with CSM and sensitized with anti-DNP IgE at 37 °C for 1 h. Then cells were washed twice with modified Tyrode's buffer supplemented with 0.1% bovine serum albumin (BSA) and 10 mM HEPES, pH 7.2, and resuspended in this supplemented buffer at a density of 0.6×10^6 cells/mL. Cells were aliquoted in 96-well plates (3×10^4 cells/well) and activated with indicated concentrations of DNP-HSA for 30 min. The release of β-hexosaminidase was determined as described before [28].

Cytokine and chemokine assays

TNF-α, IL-6, CCL3 and CXCL2 protein concentrations in the supernatants of samples were quantified using ELISA kits according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

Cells were sensitized with anti-DNP IgE and activated with CSM (1.5%) and 100 ng/mL of DNP-HSA or only with CSM for 10 min. After washing in ice-cold PBS two times, cells were solubilized by lysis buffer (containing 50 mM Tris, 110 mM NaCl, 5 mM EDTA and 1% Triton X-100, pH 8.0) and 1 mM PMSF, 100 µM Na₃VO₄, 2 mM *p*-nitrophenyl phosphate (PNPP) and 210 mU/mL aprotinin. The resuspended pellet was incubated on ice for 30 min, followed by centrifugation at 8000 g for 10 min at 4 °C. The resulting supernatants were assayed for protein concentration by BCA assays (Pierce, Perbio Science, Etten-Leur, the Netherlands) and 500 µg of each sample was subjected to immunoprecipitation with Syk antibody (Tebu-bio) and immunoblotting methods with PY20 (BD Pharmingen) as described before [30].

Preparation of nuclear extracts

Cells were washed twice with PBS and allowed to equilibrate for 5 min in ice-cold cytoplasmic extraction reagent I (Pierce) containing protease inhibitors cocktails (Roche Diagnostics BV, Almere, the Netherlands). Cells were

further lysed with cytoplasmic extraction reagent II on ice for 5 min. Following centrifugation at $3500\times g$ for 5 min, the supernatants (cytoplasmic extracts) were collected and frozen at -70°C . The pellets were suspended in nuclear extraction buffer containing protease inhibitors. After vigorous mixing and incubating for 10 min on ice, the solution was clarified by centrifugation at $14\,000\times g$ for 5 min, and the supernatant (nuclear extract) was collected and stored at -70°C . Protein concentrations were determined using a BCA protein assay kit (Pierce). Cytoplasmic or nuclear fractions (25–50 μg) were subjected to SDS/PAGE [10% (w/v) gel] for detection of I κ B- α , phospho Erk1/2, total Erk1/2, p65 and phospho-CREB/ATF-1 expression.

Western blot

The lysate (25 or 50 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 the membranes had been washed with TBS-T, antibodies against I κ B- α , p65, phospho-Erk1/2 and phospho-CREB/ATF1 were added at a dilution of 1:3000 in TBS-T and incubated according to related protocols. After three washes with TBS-T, membranes were treated for 1 h with peroxidase-conjugated relevant antibodies and diluted to 1:20 000 in TBS-T. After three washes with TBS-T, immunoreactive protein bands were revealed with an enhanced chemiluminescence (ECL) Western blot analysis system (Amersham). Films were scanned and analysed on a GS7-10 Calibrated Imaging Densitometer equipped with Quantity One v. 4.0.3 software (Bio-Rad).

Statistical analysis

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

Results

CSM does not change the surface expression of Fc ϵ RI on mast cells

Expression of the Fc ϵ RI on mast cell was analysed using flow cytometry. No effect of CSM pre-treatment on Fc ϵ RI expression on mast cells was found (Fig. 1).

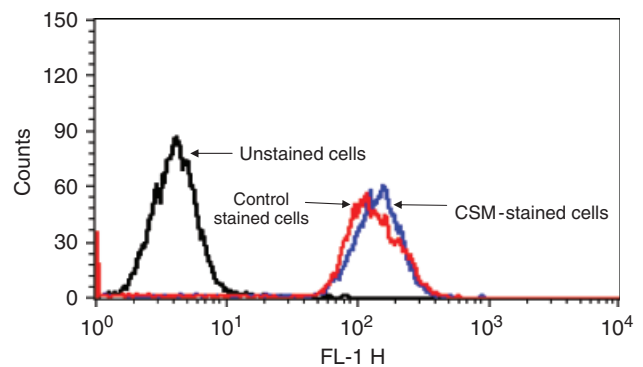


Fig. 1. Cigarette smoke medium (CSM) does not change the surface expression of the Fc ϵ RI receptor. Bone marrow-derived mast cells (BMMC) were exposed to CSM (1.5%) for 1 h and then sensitized with DNP-specific IgE, followed by activation with dinitrophenyl-conjugated human serum albumin (DNP–HSA) (10 ng/mL) or only with CSM (1.5%). Surface expression of Fc ϵ RI detected by incubation of samples with FITC-conjugated Fc ϵ RI or isotype control for 30 min. Cells were subsequently analysed using a FACS Calibur flow cytometer.

CSM suppresses degranulation, but not leukotriene production by mast cells

We determined the effect of CSM on IgE/Ag-induced degranulation and leukotriene production by mast cells. Activation with IgE/Ag caused rapid degranulation of mast cells (Fig. 2a) and exposure to CSM for 1 h considerably reduced degranulation of mast cells (Fig. 2a). Pre-incubation with NAC prevented the inhibitory effects of CSM on degranulation of mast cells. No effect of CSM exposure was seen on IgE/Ag-mediated leukotriene (LTC₄) release (Fig. 2b).

To understand the mechanism by which CSM may influence degranulation, we studied the effect of CSM on tyrosine phosphorylation of Syk. Activation of BMMC by cross-linking of IgE resulted in rapid tyrosine phosphorylation of Syk (Fig. 2c lane 2) compared with unstimulated cells (lane 1). Treatment of BMMC with CSM before IgE cross-linking resulted in considerably diminished phosphorylation of Syk after 10 min (Fig. 2c, lane 4). CSM itself did not affect Syk phosphorylation (lane 3).

CSM attenuates cytokine release but not chemokine production upon activation

To test the effect of CSM on the production of TNF- α and IL-6, BMMC were treated with CSM and simultaneously stimulated with IgE/Ag or LPS for 5 h or 16 h. CSM (1.5%) suppressed the production of TNF- α and IL-6 upon activation with IgE/Ag (Figs 3a and b). The inhibiting effect of CSM was specific for IgE-antigen-mediated activation; no suppression of cytokine production was seen in LPS-activated mast cells (Figs 3a and b). CSM by itself also increased CCL3 and CXCL2 production. In contrast to its inhibitory effect on TNF- α and IL-6 production, CSM did

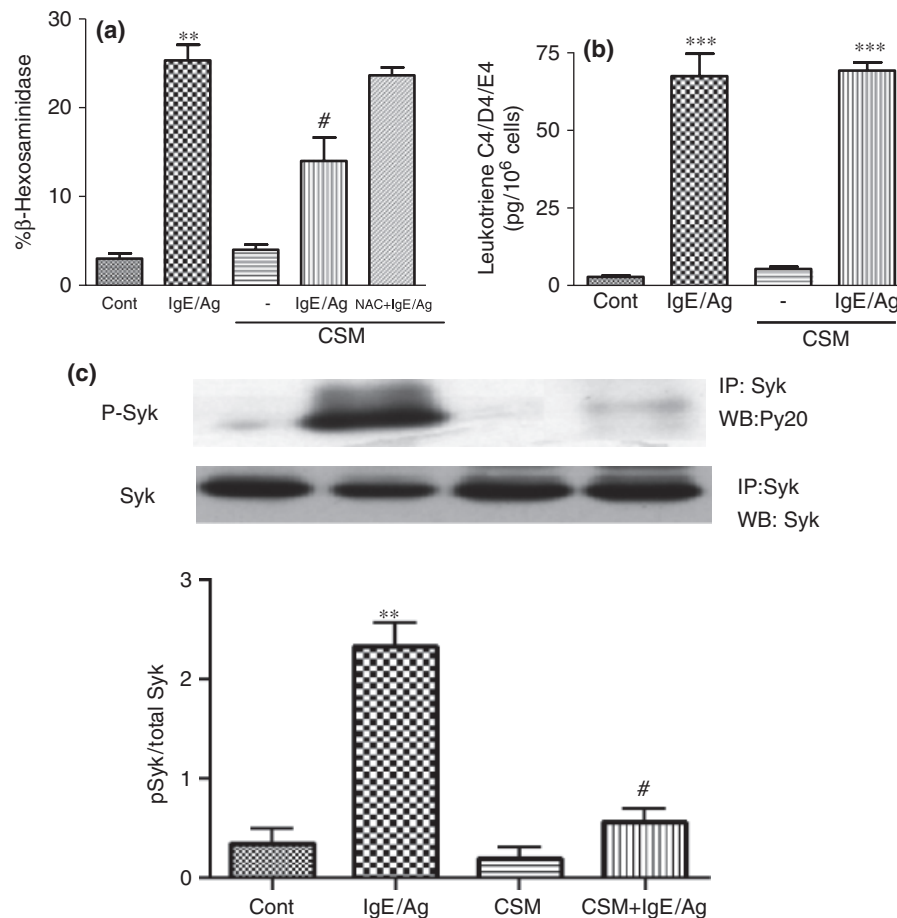


Fig. 2. Cigarette smoke medium (CSM) inhibits degranulation, but not leukotriene release from IgE/Ag-activated mast cells via the Syk pathway. Bone marrow-derived mast cells (BMMC) were pretreated with NAC (10 mM) and then exposed to CSM (1.5%) for 1 h and then sensitized with DNP-specific IgE, followed by activation with dinitrophenyl-conjugated human serum albumin (DNP-HSA) (10 ng/mL) or only with CSM (1.5%). Degranulation was assessed by the release of β -hexosaminidase (a) and cysteinyl leukotrienes (b) using an enzyme immunoassay in the supernatants from activated cells. The percentage of degranulation was calculated as: $(a-b)/(t-b) \times 100$, where a is the amount of β -hexosaminidase released from stimulated cells, b is that of β -hexosaminidase released from unstimulated cells and t is the total cellular content of β -hexosaminidase. Data are mean $\pm$ SEM of quadruplicate samples. The asterisks represent significant differences compared with activated cells alone (** $P < 0.01$, *** $P < 0.001$) or non-activated controls. Hatches represent significance between activated cells with IgE/Ag and IgE/Ag with CSM (# $P < 0.05$). (c) BMMC were treated with CSM and then stimulated with IgE and antigen as described. Cells were lysed, and Syk was immunoprecipitated from the supernatants using antibodies specific for Syk. Proteins were resolved by SDS-PAGE and analysed by Western blotting (WB) with anti-phosphotyrosine mAb PY-20 conjugated with HRP. After stripping, mAbs specific for Syk were used to estimate the relative amounts of the immunoprecipitated (IP) proteins. Densitometric analysis of three immunoblots from three independent experiments of tyrosine-phosphorylated Syk to total Syk is shown in the lower panels. Data are mean $\pm$ SEM. The asterisks represent significant differences compared with non-activated cells alone (** $P < 0.01$), and hatches indicate significant differences between IgE/Ag alone and IgE/Ag and CSM (** $P < 0.01$).

not affect LPS and IgE/Ag-induced production of chemokines CCL3 and CXCL2 (Figs 3c and d).

Effects of CSM on I κ B- α degradation, p65 expression and Erk1/2, CREB/ATF-1 phosphorylation

Activation of mast cells with IgE/Ag and LPS stimulates phosphorylation of mitogen-activated protein kinase (MAPK) [31–33] and activates nuclear factor (NF)- κ B pathways. Recently, we have demonstrated that CSM itself did not stimulate degradation of I κ B- α in mast cells [27]. We further explored whether CSM attenuated cytokine produc-

tion of mast cells upon IgE/Ag activation via the NF- κ B and MAPK pathways. I κ B- α degradation and phosphorylation of Erk1/2 were analysed by Western blotting of whole-cell extracts with specific Abs. As shown in Fig. 4a, activation with IgE/Ag and LPS resulted in a reduction of I κ B- α levels. CSM abrogated I κ B- α degradation in response to IgE/Ag, while LPS-induced I κ B- α degradation was not inhibited (Fig. 4a). In nuclear fractions, we studied the presence of NF- κ B (p65). As shown in Fig. 4b, upon activation with LPS, IgE/Ag nuclear translocation of p65 was increased. Pre-incubation of cells with CSM suppressed the translocation of p65 induced by IgE/Ag but not by LPS (Fig. 4b).

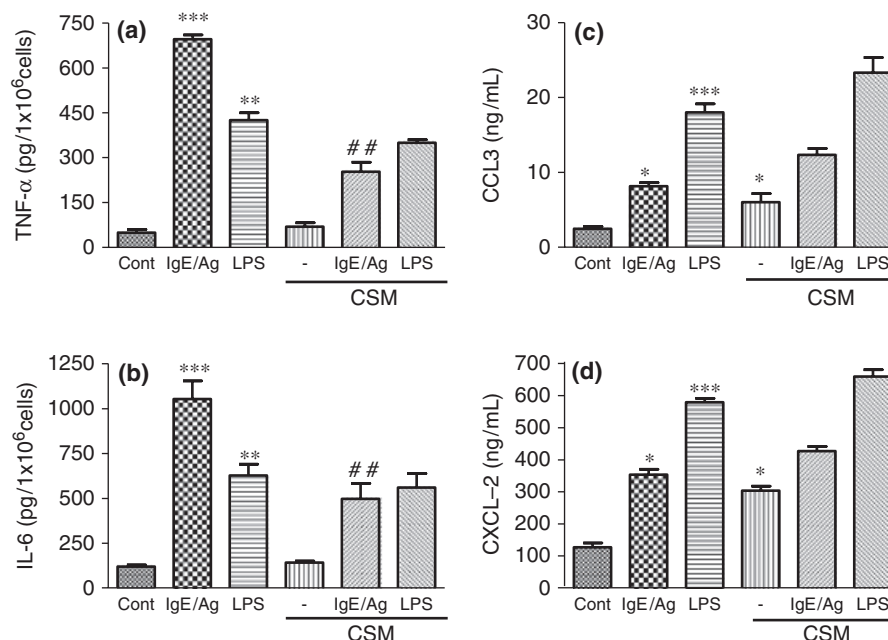


Fig. 3. Cigarette smoke medium (CSM) modulates cytokine but not chemokine production by IgE/Ag-activated mast cells. Bone marrow-derived mast cells (BMMC) were sensitized with DNP-specific IgE for 1 h, followed by activation with dinitrophenyl-conjugated human serum albumin (DNP-HSA) (10 ng/mL) alone or with CSM (1.5%), LPS (100 ng/mL) for 5 h for detection of cytokines or 16 h for chemokine release. The supernatants of the samples were tested for TNF- α (a), or IL-6, (b) CCL3, (c) and CXCL2 (d) production by ELISA. Data shown are mean $\pm$ SEM of three independent experiments. The asterisks represent significant differences between activated cells and non-activated controls (** P < 0.01 and *** P < 0.001). Hatches indicate significant differences between IgE/Ag alone and IgE/Ag and CSM (** P < 0.01).

Moreover, as shown in Fig. 4c, exposure to CSM increased phosphorylation of Erk1/2 in the cytoplasm. While no further increase of Erk1/2 phosphorylation was found upon activation with IgE/Ag, LPS induced an additional increase in phosphorylation of Erk1/2 in CSM-treated mast cells.

In nuclear fractions, we studied the presence of CREB/ATF-1. As shown in Fig. 4d, upon stimulation of cells with IgE/Ag, CSM phosphorylation of ATF-1 was increased.

Discussion

CS exposure has been reported to influence allergic immune responses. Confusingly, both attenuation and exacerbation of allergic responses have been shown, [34, 35]. Because mast cells are central regulators of allergic reactions and innate immunity, we have studied the effects of CSM on mast cells after allergic activation.

Our present data show that CSM suppressed IgE/Ag-induced mast cell degranulation as well as the release of the cytokines TNF- α and IL-6 but not chemokine (CCL3 and CXCL2) production. As LPS-induced cytokine release was not affected by CSM, we conclude that the effect of CSM was specific for allergic activation of the mast cells. Allergic activation was induced by cross-linking of the high-affinity receptor for IgE (Fc ϵ RI) on the mast cell membrane [36]. We ruled out the possibility that CSM decreased the expression of Fc ϵ RI on the mast cell

membrane. Consequently, we hypothesized that CSM might have influenced intracellular signalling. An early event after Fc ϵ RI cross-linking is the activation of Syk [17]. Our data clearly show that CSM inhibited IgE/Ag-induced phosphorylation of Syk. Subsequent intracellular propagation of Fc ϵ RI activation, leading to the various cellular effects of mast cells, involves several possible signalling pathways. Also, in this respect CSM seemed to be specific in its effects. Because the release of neither leukotrienes nor chemokines from IgE/Ag-activated mast cells was affected by CSM, this indicates that specific signalling pathways were influenced. Indeed, we found that signalling through the MAPK Erk1/2 was not influenced by CSM, as Erk1/2 phosphorylation induced by IgE/Ag was not inhibited.

A further potential target of CSM might be the CREB/ATF family of transcription factors (downstream of Erk1/2), which has been shown to regulate proliferation and apoptosis [37]. We did not find evidence of an effect of CSM on CREB phosphorylation; however, CSM treatment potentially increased ATF-1 phosphorylation in IgE/Ag-activated cells. At present, nothing is known about the regulatory role of ATF-1 in mast cells. It has been reported for other cells that SB 203580 (as an inhibitor of p38 MAPK) inhibits phosphorylation of CREB and ATF-1 [38, 39], implicating an involvement of p38 MAPK in their activation. In our hands, SB 203580 was not able to prevent CSM-induced attenuation of cytokine release by

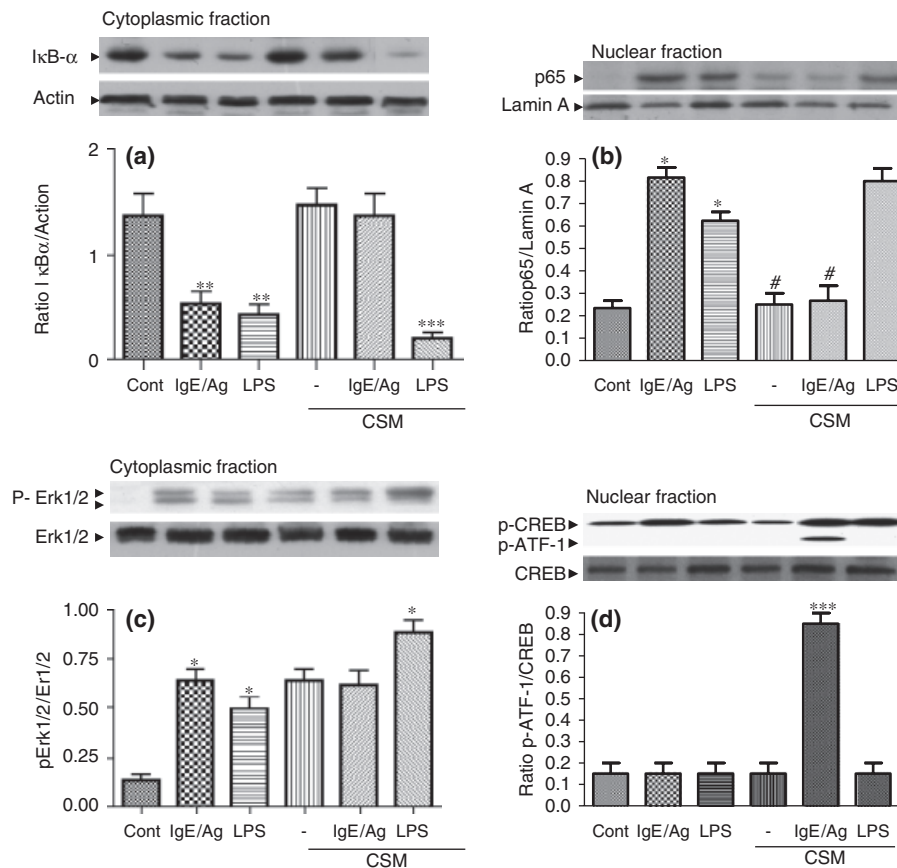


Fig. 4. Cigarette smoke medium (CSM) modulates NF- κ B and CREB pathways. BMMC were pre-treated with CSM (1.5%) and activated with IgE and antigen or LPS (100 ng/mL) for 30 min, as described in 'Materials and methods'. Western blot analysis of I κ B- α and p-Erk1/2 in cytoplasmic fractions (a and c) and p65 and p-CREB/ATF-1 in nuclear fractions (b and d) was carried out with specific antibodies. Actin and Erk1/2 served as loading controls for the cytoplasmic fraction and lamin and CREB served as controls for nuclear fractions. The data are representative for three independent experiments. Densitometric analyses of immunoblots from three experiments are shown in the lower panels (mean $\pm$ SEM). The asterisks represent significant differences compared with activated cells alone and activated cells with CSM (* P < 0.05, ** P < 0.01 and *** P < 0.001), and hatches indicate significant differences between IgE/Ag alone and IgE/Ag and CSM (** P < 0.01).

IgE/Ag-activated mast cells (data not shown), suggesting that either ATF-1 in mast cells is activated by other MAPK members or that ATF-1 does not mediate CSM effects. However, when we take into consideration that CSM did not increase ATF-1 phosphorylation in LPS-activated mast cells, and that CSM did not inhibit LPS-induced degranulation and cytokine release, it is tempting to speculate that ATF-1 phosphorylation is indeed involved in CSM effects in mast cells after allergic stimulation.

Another important intracellular signal transduction pathway in mast cells involves the transcription factor NF- κ B that regulates the expression of many pro-inflammatory cytokines [40, 41]. In mast cells, NF- κ B is a heterodimer composed of the RelA (p65) and NF- κ B1 (p50) subunits [41]. NF- κ B is maintained as an inactive complex in the cytoplasm when bound to inhibitory I κ B proteins [41]. Activation of NF- κ B involves phosphorylation of I κ B by an I κ B kinase and its subsequent breakdown, followed by translocation of NF- κ B to the nucleus.

We found that CSM suppressed the IgE/Ag-induced decrease of cytosolic I κ B- α , while it did not affect LPS-induced I κ B- α decrease. The concomitant increase of NF- κ B (p65) in the nucleus by IgE/Ag was likewise inhibited by CSM. Again, LPS-induced NF- κ B translocation to the nucleus was not inhibited by CSM. These findings clearly demonstrate that CSM inhibits the NF- κ B signalling pathway in mast cells, but only after allergic activation, which may thus account for the observed inhibition of cytokine release.

In conclusion, this study shows that CS may reduce allergic activation of mast cells, resulting in decreased degranulation and cytokine production. These effects seem to be mediated by the NF- κ B signal transduction pathway and, possibly, through the ATF-1 transcription factor. Further research is needed to determine whether this inhibitory effect of CS on activation of mast cells may contribute to a decrease in allergic symptoms in smokers.

Acknowledgement

Competing interests:

The author(s) declare that they have no competing interests.

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