

# In Vitro Exposure To Cigarette Smoke Activates Eosinophils: Implications For Lung Inflammation

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## Citation

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## Abstract

Effects of cigarette smoke (CS) on eosinophils (EOS), important cells involved in the pathogenesis of chronic lung diseases such as asthma, were studied in vitro. EOS were isolated from healthy and mildly atopic donors and exposed to soluble components of cigarette smoke (CSE). Viability and apoptosis were assessed by flow cytometry after staining with propidium iodide and Annexin-V. Activation was determined by release of newly-synthesized (IL-8, IL-6) mediators and by phosphorylation of MAPKs. CSE effects on ultrastructural morphology and production of neutrophil chemotactic factors in CSE-activated EOS were also evaluated. CSE concentrations from 0-2.5% were non-toxic for up to 18-24 hours of exposure. However, CSE at 2.5% activated EOS as evidenced by ultrastructural degranulation: release of IL-8 and IL-6, and increased expression of the MAPK, c-Jun. Supernatants from CSE-activated EOS were found to be significantly chemotactic for neutrophils. These results suggest that CS may aggravate lung inflammation by activating EOS which, in turn, release inflammatory mediators promoting inflammatory cell recruitment and lung remodeling.

## INTRODUCTION

Eosinophils (EOS) are terminally differentiated granular leukocytes that are produced in the bone marrow and migrate to inflamed tissues in response to chemotactic signals. Their recruitment, growth and survival are supported by cytokines and chemokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukins (IL)- 3 and -5 [1,2,3,4]. Eosinophilia occurs in response to parasitic infection and exposure to allergens. Eosinophils are particularly evident in chronic lung inflammation where their products appear to play roles both in bronchial hyper-responsiveness and in tissue remodeling, leading to compromised lung function [5,6]. Increased numbers of eosinophils in the bronchial mucosa have been correlated with a number of conditions such as: bronchial wall thickening, epithelial cell hypertrophy, and myofibroblast hyperplasia [7]; Goblet cell hyperplasia and increased mucus production [7]; and tissue remodeling [8]; and fibrosis [9]. Neutralization of IL-5, the main eosinophil growth factor, has been shown to block airway hyper-reactivity [10].

Many of the eosinophil mediators involved in the pathogenesis of airway inflammation are preformed and

stored in granules that are released upon eosinophil activation. These include: major basic protein (MBP) whose levels are correlated with bronchial epithelial damage [11]; eosinophil cationic protein (ECP) which has been shown to be elevated in chronic asthma and associated with airflow obstruction [12]; eosinophil-derived neurotoxin (ECN) which stimulates fibroblast proliferation [13]; and eosinophil peroxidase (EPO) which catalyzes the peroxidation of halides and forms toxic nitrogen reactive species that contribute to asthmatic inflammation [14]. EPO, which is used as a biomarker of eosinophil degranulation (i.e., activation) [15], inactivates leukotrienes that cause bronchoconstriction [16]. MBP, the main granule constituent also induces degranulation of tissue mast cells, which contribute to airway hyper-responsiveness and inflammation [17]. Additionally, eosinophil cytoplasmic lipid bodies contain cyclooxygenases (COX), lipoxygenases, and phospholipase A2, all of which contribute to inflammation through their role in synthesis of proinflammatory eicosanoids [18]. Upon activation, eosinophils also synthesize and release a variety of proinflammatory cytokines including: the interleukins IL-1, IL-6, IL-8; tumor necrosis factor- alpha (TNF- $\alpha$ ); transforming growth factors (TGF- $\beta$ ) and  $\gamma$  and eotaxin [19,20]. TNF- $\alpha$ , which is also produced by

several other inflammatory cell types, causes recruitment of eosinophils and neutrophils to inflamed tissues, induces eosinophil synthesis and release of matrix metalloproteinases (MMPs) involved in lung tissue remodeling, and synergizes with GM-CSF to promoting eosinophil survival [21]. It induces eosinophil synthesis and release of IL-8, an important chemotactic and activating factor for neutrophils and lymphocytes [22,23] which induces adhesion molecule expression also triggers the oxidative burst response, contributing to tissue damage [24]. IL-6, a highly pleiotrophic cytokine, stimulates a variety of immune and inflammatory responses and plays an important role in airway inflammation [25,26,27]. Eosinophil-derived TGF- $\beta$  promotes lung fibrosis by stimulating fibroblast proliferation and synthesis of extracellular matrix (ECM) components such as collagen [28]. Thus, intrinsic agents (such as TNF- $\beta$  and C5a) and extrinsic agents which persistently activate airway eosinophils may induce an inflammatory cascade leading not only to chronic inflammation but also lung tissue remodeling and compromise of lung function.

The adverse effects of long term exposure to cigarette smoke (CS) on human health are increasingly being documented both in human and animal studies. CS exposure is associated with reduced immune function [29,30]. Exposure to CS also influences inflammatory responses by enhancing release of proinflammatory cytokines [31,32]. CS is composed of a multitude of potentially harmful chemicals including aldehydes (formaldehyde, acrolein), ketones, organic acids, phenols, cyanides, nitrogen oxides (NO) and reactive oxygen species (ROS); thus, exposure to CS likely induces a state of oxidative stress leading to release of proinflammatory cytokines such as TNF- $\beta$ , IL-6 and IL-8 through activation of stress kinases such as JNK and p38 and redox-sensitive transcription factors such as NF- $\beta$  and AP-1 [33]. Other CS components such as formaldehyde and acrolein enhance neutrophil migration into the lung [34] and inhibit apoptosis of neutrophils [35] and high concentrations of nicotine stimulate neutrophil degranulation [36]. In both humans and animals, CS induces release of chemokines such as monocyte chemoattractant protein (MCP)-1 and IL-8 that attract monocytes/macrophages and neutrophils to the lung [37]. In vitro exposure to CS directly activates human bronchial epithelial cells, lung fibroblasts, and alveolar macrophages resulting in release of inflammatory mediators such as IL-8 [32,38,39,40]. Levels of TNF- $\beta$  and IL-6 are often found to be elevated in the lungs of smokers [41]. While these proinflammatory cytokines may have several cellular

sources (in addition to eosinophils) observations in animal models and humans suggest that CS may influence survival, recruitment, and activation of eosinophils. Guinea pigs repeatedly exposed to CS demonstrated eosinophilic infiltration in the lung mucosa [42]. Observations of increased airway remodeling in asthmatic smokers in comparison to asthmatic non-smokers [43], reduced peripheral blood eosinophil counts [44] and increased numbers of eosinophils in bronchial lavage fluids of (BALF) smokers [45] suggest that these inflammatory cells may also be affected by CS exposure. However, other than what has been described above little is known about the effects of CS on eosinophil survival and functional status. We undertook a preliminary investigation to examine the effects of CS exposure in vitro on eosinophil survival, response to activating agents such as TNF- $\beta$  and C5a, and their elaboration of proinflammatory cytokines – in particular, IL-6 (which has been shown to be elevated in the lungs of smokers and animals exposed to CS [20,41,42,45]), and IL-8, a major chemotactic factor for neutrophils which may be affected by exposure to CS [32].

### **MATERIALS AND METHODS.**

**Production of cigarette smoke extract (CSE):** Smoke from one cigarette (Marlboro Lights, Phillip Morris, estimated to contain 10 mg tar, 0.8 mg, 11 mg carbon monoxide at the smoker end [46]) was extracted under vacuum for 5 min into 10 ml of culture medium using an apparatus designed for this purpose as previously described [47]. The solution was subsequently sterilized through a 0.45 m disposable filter. The cigarette smoke extract (CSE) was prepared freshly for each experiment and assigned an absolute value of 100%

**Purification of eosinophils from peripheral human blood:** Heparinized blood (150 ml) was collected by venipuncture from non atopic and atopic volunteers. Informed consent was obtained from all volunteers according to the guidelines established by the Hadassah- Hebrew University Human Experimentation Helsinki Committee. Samples were treated and tested separately. The blood was mixed with dextran (50 ml blood: 10 ml dextran) and erythrocytes were allowed to sediment for 45 min at 37°C. The buffy coat was collected, layered over Ficoll-Hypaque (Histopaque® -1077, 1.77g/ml, Sigma-Aldrich, St. Louis, MO); granulocytes were separated by density gradient centrifugation performed at room temperature. The pellet was collected and residual erythrocytes were removed by hypotonic lysis, leaving a population consisting of neutrophils and eosinophils. Eosinophils were separated from neutrophils by negative immunomagnetic selection with a magnetic cell separation

column (Magnetic cell separation [MACS] column, Miltenyi Biotec, Sunnyvale, CA) using anti-CD16 and anti-CD3 magnetic beads to remove neutrophils and lymphocytes/monocytes, respectively. Cytospins of the cells in the effluent were stained with Kimura solution to identify eosinophils. The eosinophil content of these preparations was greater than 95% in all experiments.

**Eosinophil cultures:** Freshly isolated eosinophils were seeded into 96-well culture plates at a concentration of  $2 \times 10^5$  cells/200  $\mu$ l medium and cultured at 37°C in humidified atmosphere of 5% CO<sub>2</sub> for 1 min to 48 hours, according to the different experimental aims. For all experiments RPMI-1640 medium (Biological Industries, Beit HaEmek, Israel) supplemented with 10% heat-inactivated foetal calf serum (FCS, Biological Industries) was used. According to the experimental protocol, CSE (0-10%) was added to medium. Concentrations of 1% and 10% were considered equivalent to smoking 4 cigarettes/day and 2 packs (20cigarettes /pack) of cigarettes/day, respectively.

**Eosinophil activation and mediator release assays:** Eosinophils ( $2 \times 10^5$  cells/200  $\mu$ l) were cultured in the presence of 5000 units (previously shown to be optimal for activation of eosinophils) of recombinant human TNF $\alpha$  (Cytolab, Rehovot, Israel) with or without the addition of GM-CSF [10ng/ml] for 18, 24, or 48h according to the experimental aim. Viability and apoptosis were assessed at 18, 24 or 48h; cytokine release and ultrastructural morphological changes were evaluated after 18h; electronmicroscopy; and signal transduction measurements were made during the period spanning 0-360 min after activation. Immediately following culturing, eosinophils were analyzed for apoptosis and viability by Annexin V-propidium iodide (PI) (see below) staining. Supernatants were stored at -70°C until analyzed for cytokine content by ELISA (see below). Eosinophils were stored at +4°C either for intracellular FACS staining or electronmicroscopy.

**Assessment of cell death by Annexin V-FITC and propidium iodide double staining:** The proportion of cells undergoing apoptosis and dead cells were measured at the indicated times (18 h, 24h, and 48h) by Annexin V-FITC [10 $\mu$ l per  $1 \times 10^6$  cells]-PI [5 $\mu$ g/ml] double-staining (both reagents were purchased from Sigma-Aldrich). Eosinophils [ $2 \times 10^5$ ] were washed in Annexin V buffer and then incubated for 20 min with Annexin V-FITC according to a standard protocol established in our laboratory [72]. Cells washed again and PI was added and subsequently analyzed by flow cytometry

(FACScalibur, Becton Dickenson, San Jose, CA).

**Electron microscopy:** Eosinophils were fixed in Karnovsky's fixative solution and processed as previously described [48]. Sections were observed with a Joel 100CX transmission electron microscope (TEM) and with a Philips CM12 TEM equipped with a goniometer. Eosinophils were photographed at random. These studies were performed in the laboratory of Professor Ilan Hammel (Department of Pathology, Tel Aviv University, Tel Aviv, Israel).

**Measurement of IL-8 and IL-6 supernatants from cultured eosinophils:** Concentrations of IL-8 and IL-6 in culture supernatants were measured using commercial ELISA kits (IL-8 DuoSet ELISA development kit, IL-6 DuoSet ELISA development kit, both from R&D Systems, Minneapolis, MN, USA) and according to the manufacturer's instructions.

**Neutrophil transmigration assay:** Neutrophil transmigration was measured using a microwell dual chamber system (Chemotex Microwell dual chamber system, filter pore size 5  $\mu$ m, 6.0 mm diameter wells, from NeuroProbe Inc., Gaithersburg, MD). Samples from individual donors were treated separately for both eosinophil and neutrophil populations. Supernatants of eosinophils cultured for 18h in medium alone, or in medium containing 2.5% of CSE, were first added in triplicates to wells in the bottom chamber and covered with a framed filter. Next, neutrophils [30,000 cells in 30 $\mu$ l of culture medium] were placed on the top of the filter over each well and the chamber system was incubated for 90 min [37°C, 5% CO<sub>2</sub>] to allow neutrophils to migrate through the filter in response to chemotactic factors present in the well. After incubation, the remaining neutrophil suspensions on the top of the filter were removed using tissue paper and plate was centrifuged [for 1 min at 600 g] to collect cells attached on the bottom of the filter. Then, cells in the lower chamber were enumerated by flow cytometry by acquiring events for 1 min according to a standard protocol established in our laboratory [71]

**Intracellular staining for flow cytometric analysis of protein expression:** Eosinophils were activated (as described above) and exposed to 2.5% CSE, then immediately fixed [15 min, room temperature] at the indicated time points [0-360 min] in 4% paraformaldehyde in Hanks Balanced Salts solution (Biological Industries) containing bovine serum albumin [0.1% w/v] and sodium azide [3 mM] (HBA) followed by permeabilization [30 min on ice] in HBA containing saponin [0.1%], BSA [1mg/ml] and human serum [10%]. Primary antibodies: rabbit anti-phospho ERK1/2 (Biosource,

Camarillo, CA, USA); mouse anti-phospho P38 (BD Biosciences, San Jose, CA, USA); mouse anti phospho JNK (Cell Signaling, Danvers, MA, USA); rabbit anti-cJun (Santa Cruz Biotechnology, San Diego, CA, USA) (all 1 $\mu$ g/ml) or isotype control: -mouse IgG1 or rabbit IgG1, both at 1 $\mu$ g/ml from Dako, Glostrup, Denmark] were added to the treated cells and incubated for 30 min on ice. Specific binding of primary antibodies was visualized with FITC anti-mouse (Ansell Bayport, MN, USA., 1:250 in HBA) and Cy5 anti-rabbit (Jackson Immunoresearch Laboratories West Grove, PA, USA, 1:200 dilution in HBA) following incubation for 30 min on ice. After staining, the cells were analyzed by flow cytometry. For each staining at least 10,000 events were collected and data analysed using CellQuest software (Becton Dickenson, Mansfield, MA).

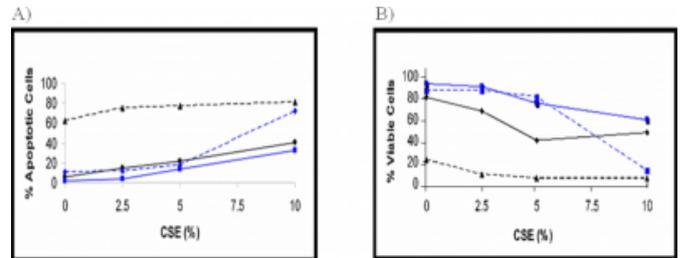
Statistical analysis: Microsoft Excel™ analysis tool-pack was used to perform statistical analyses. Mean values were compared by 2-way ANOVA. When the ANOVA probability value was significant [ $p < 0.05$ ], the control and experimental groups were compared by t-test. The data are expressed as the mean  $\pm$  standard deviation (SD) of at least three independent experiments involving cell populations from three different donors.

### RESULTS.

Effects of CSE on viability and induction of apoptosis in human eosinophils. The threshold of eosinophil sensitivity to CSE, optimal CSE working concentration and exposure time were established. As GM-CSF is the main survival factor of eosinophils the effect of CSE on GM-CSF-mediated eosinophil survival was also assessed. Viability and apoptosis were measured by AnnexinV/PI staining and flow cytometry and results are shown in Fig. 1. At CSE concentrations between 2.5% and 5% a slight increase in the rate of apoptosis was observed (Fig. 1A). After 24h of exposure to CSE, even in absence of GM-CSF no change in number of apoptotic cells was measured at CSE concentrations of  $\leq 2.5\%$ . However, after 48h of culture, 62.25% of the unexposed cells stained for Annexin V. Nevertheless, in CSE-exposed cells the percentage of cells undergoing apoptosis was markedly higher at all CSE concentrations (2.5% CSE: 76%; 5% CSE: 77%; 10% CSE: 81%). At 48 hours, even in the presence of GM-CSF, a dramatic increase of apoptotic cells from 11% to 72% was observed.

### Figure 1

Figure 1: Exposure to CSE reduces eosinophil viability. Eosinophils [ $2 \times 10^6$ ] were cultured for 24 h (solid line) and 48 hours (dotted line) in medium alone, or with different concentrations of CSE [2.5-10%] in the presence (blue lines) or absence (black lines) of GM-CSF [20 ng/ml]. Cells were stained with Annexin V/PI and analyzed by flow cytometry. Results shown are representative of three separate experiments: Cells undergoing apoptosis.: Viable cells.



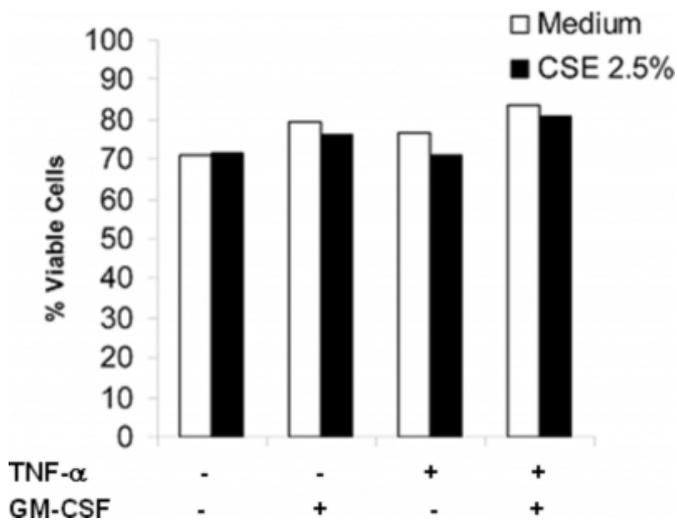
In the absence of GM-CSF, eosinophil survival was reduced over time in culture regardless; however, exposure to CSE for 24-48h contributed significantly to the decline in eosinophil viability at all CSE concentrations tested (Fig. 1B). In the presence of GM-CSF, eosinophil viability was not markedly affected by exposure to CSE concentrations of  $\leq 2.5\%$  for 24- 48h. However, this was not the case at [CSE]  $> 2.5\%$ . Thus, at lower CSE concentrations (i.e., 2.5% and lower) the addition of the survival factor, GM-CSF appeared to partially rescue eosinophils from the additional toxic effects of CSE. It also appeared that the interval between 12-24h was a critical determinant of eosinophil survival in the absence of GM-CSF and after exposure to CSE.

Exposure to CSE at concentrations of 2.5% for 18 hours does not adversely affect the viability of eosinophils. Based on the results described above 2.5% CSE was chosen as the optimal concentration. At this concentration, eosinophil survival was neither adversely affected nor improved by addition of GM-CSF. However, at higher CSE concentrations, eosinophil survival was decreased even in the presence of GM-CSF. Thus, we determined the optimal exposure time for 2.5% CSE concentration and its affect on eosinophil activation with agents such as TNF- $\alpha$ . Eosinophils were incubated in the presence of 2.5% CSE for 18h. In these experiments untreated cells were compared to CSE (2.5%)-treated cells incubated in the presence or absence of TNF- $\alpha$  [5000 U] or GM-CSF [20 ng/ml]. Viability was determined by PI staining and flow cytometry and results are shown in Fig. 2. Exposure to 2.5% CSE did not affect eosinophil viability after 18h and addition of GM-CSF or TNF- $\alpha$  did not influence the outcome with respect to eosinophil viability. Based on these results, in subsequent

experiments eosinophils were cultured for no longer than 18h and at CSE concentrations not exceeding 2.5%.

**Figure 2**

Figure 2: Exposure to CSE at concentrations of 2.5% for 18 hours does not adversely affect the viability of eosinophils. Eosinophils [ $2 \times 10^6$ ] were cultured for 18 h in either medium alone, or with 2.5% of CSE in the presence or absence of TNF- $\alpha$  [5000U] or GM-CSF [20 ng/ml]. Cells were stained with Annexin V and PI and analyzed by flow cytometry for relative numbers of apoptotic and viable cells. Results shown are representative of three separate experiments.

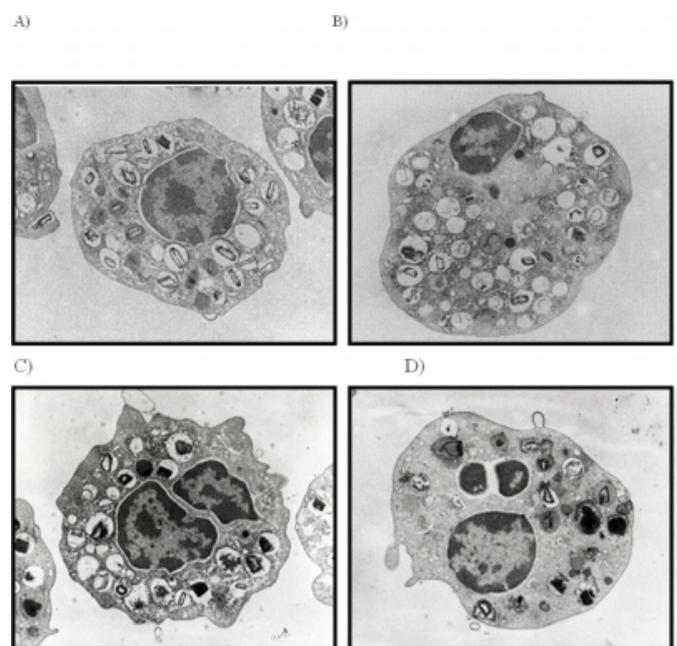


Exposure to 2.5% CSE for 18h induces ultrastructural morphological changes in eosinophils. To determine whether CSE induces ultrastructural changes in eosinophils exposed to 2.5% CSE for 18h were fixed, sectioned, and examined by transmission electron microscopy and these were compared to cells incubated for the same period of time in medium without CSE. A representative electron micrograph of untreated eosinophils cultured for 18h is shown in Fig. 3A. In this cell some granules are electron-transparent suggesting partial degranulation (“piecemeal degranulation”) while the majority of granules appear normal, containing core protein, visible as a large crystal structure within the granule. The CSE-exposed eosinophil shown in Fig. 3B appears enlarged in comparison to the untreated cell (Fig. 3A) and most of its granules are electron transparent, lacking the core crystal. Its cytoplasm contains numerous vesicles and an area of “cloudy swelling” suggestive of hydropic degeneration due to cellular stress. Electron micrographs of cells that were incubated with GM-CSF [10ng/ml] and TNF- $\alpha$  [5000U] are shown in Figs. 3C and 3D. In the eosinophil incubated with GM-CSF and TNF- $\alpha$ , but not exposed to CSE (Fig. 3C), the granule content and the crystal core appear more electron-dense than the untreated control eosinophil (Fig. 3A)

suggesting that GM-CSF and TNF- $\alpha$  support cell viability and stability despite the physiologic stress imposed by culture conditions. Its cytoplasm has a vesicular appearance suggesting “piecemeal degranulation” as a result of TNF- $\alpha$  activation of the cell. While the eosinophil shown in Fig. 3D was also exposed to 2.5% CSE for 18h the majority of its granules contain their crystal core, suggesting that in the presence of GM-CSF and TNF- $\alpha$  eosinophil structure is more preserved. However, in some granules the crystals appear partially dissolved and many vesicles are scattered within the cytoplasm suggesting that CSE is enhancing “piecemeal degranulation” in response to the culture conditions. Thus, while the culture conditions activate eosinophils to some degree, exposure to CSE appears to augment eosinophil activation as evidenced by loss of granule contents.

**Figure 3**

Figure 3: Exposure to 2.5% CSE for 18h induces morphological changes in eosinophils. Shown are transmission electron micrographs of eosinophils cultured for 18 h either in the presence or absence of 2.5% CSE.: Unexposed, untreated eosinophil.: CSE-exposed cell.: Cell exposed to GM-CSF [10 ng/ml] and TNF- $\alpha$  [5000U] without CSE. : Cell exposed to 2.5% CSE in the presence of GM-CSF and TNF- $\alpha$ .

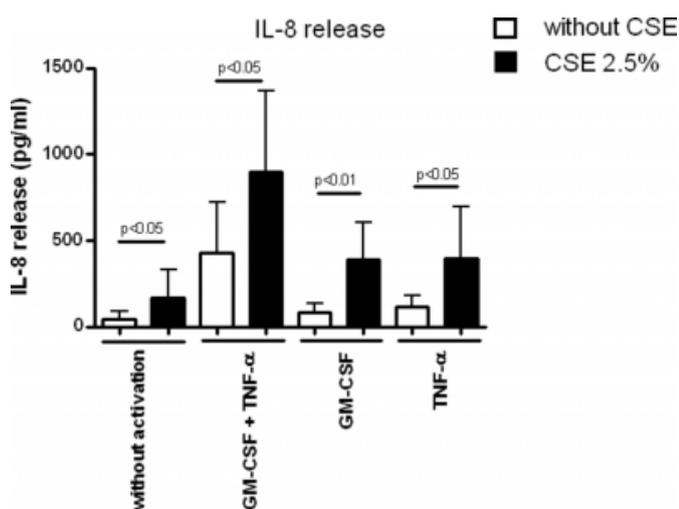


Exposure to CSE in vitro increases release of IL-8 and IL-6 from eosinophils. IL-8 and in particular, IL-6 play an important role in CS-induced lung injury as well as in asthma and it is known that in vitro stimulation of eosinophils with GM-CSF and TNF- $\alpha$  can induce their synthesis [22]. TNF- $\alpha$  is one of the mediators that are increased in the lung microenvironment following CS-

induced lung injury. As exposure of eosinophils to CSE differentially affected degranulation in activated eosinophils (described above), the possibility that it would also affect pro-inflammatory cytokine release by GM-CSF/TNF- $\alpha$ -activated eosinophils was also investigated. To exclude the possibility that CSE affects only the release and not the de novo synthesis of these cytokines, the protein synthesis inhibitor cycloheximide was included as a control in parallel cultures for all treatments. IL-8 and IL-6 concentrations in eosinophil culture supernatants were determined by ELISA. IL-8 concentrations (Fig. 4) in supernatants of eosinophils cultured in the presence of 2.5% CSE (without GM-CSF and TNF- $\alpha$ ) were significantly higher in comparison to those in supernatants of cells not exposed to CSE ( $378 \pm 18$  pg/ml vs.  $105 \pm 25$  pg/ml  $p < 0.02$ ). IL-8 release into culture supernatants of eosinophils activated with GM-CSF + TNF- $\alpha$  then exposed to 2.5% CSE was significantly higher in comparison with eosinophils activated in medium alone ( $975 \pm 10$  pg/ml vs.  $362 \pm 13$  pg/ml  $p < 0.02$ ).

**Figure 4**

Figure 4: Exposure to CSE increases release of IL-8 from eosinophils. Eosinophils [at 10 cells/ml] were exposed to 2.5 % CSE for 18h either in the presence or absence of TNF- $\alpha$  [5000U/ml] and/or GM-CSF [10 ng/ml], and either with or without cycloheximide [0.1mg/ml] to inhibit protein synthesis. IL-8 release into culture supernatants was measured by ELISA. Results are expressed as the mean + SD of IL-8 concentration (pg/mL) in culture supernatants and are representative of six experiments performed in triplicate.

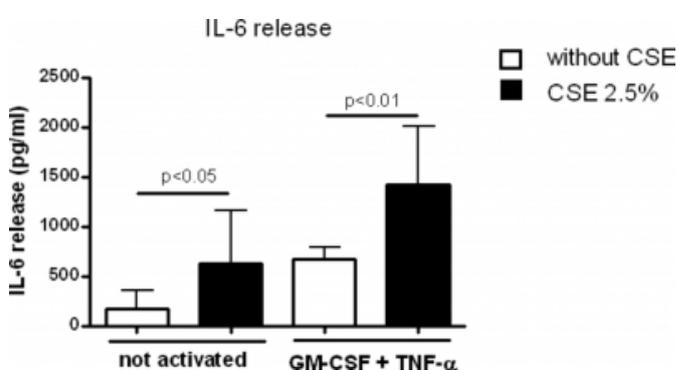


Concentrations of IL-6 (Fig. 5) in supernatants of eosinophils cultured in the presence of 2.5% CSE were also significantly higher in comparison to those in supernatants of cells cultured in medium alone ( $400 \pm 67$  pg/ml vs.  $1329 \pm 302$  pg/ml  $p < 0.05$ ). In eosinophils activated with GM-

CSF + TNF- $\alpha$  and exposed to 2.5% CSE, IL-6 release was significantly increased ( $770 \pm 19$  pg/ml vs.  $2174 \pm 610$  pg/ml  $p < 0.05$ ) in comparison with eosinophils activated in medium alone. This effect on IL-8 and IL-6 release was not observed in cycloheximide-treated eosinophils, suggesting that CSE influences de novo synthesis of these cytokines.

**Figure 5**

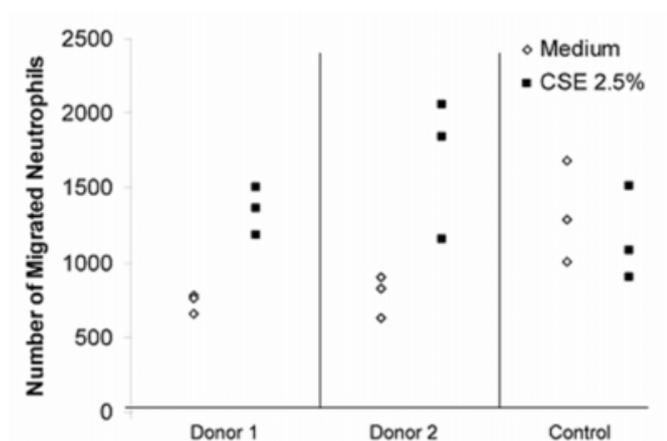
Figure 5: CSE increases release of IL-6 from eosinophils. Eosinophils [10 cells/ml] were incubated with 2.5 % CSE in the presence or absence of TNF- $\alpha$  [5000U/ml] and GM-CSF [10 ng/ml]. IL-6 release into culture supernatants was measured by ELISA and the results are expressed as pg/ml. Results shown are the mean  $\pm$  SD of three separate experiments in which samples were tested in triplicate.



Supernatants from CSE-activated eosinophils enhance neutrophil chemotaxis. Neutrophils play a major role in lung inflammation induced by cigarette smoke exposure. Soon after exposure of lung to cigarette smoke an influx of neutrophils into the lung has been observed [37] and IL-8 is a major chemoattractant for neutrophils [49]. As exposure of eosinophils to 2.5% CSE induced synthesis and release of IL-8, the possibility that CSE might indirectly induce neutrophil chemotaxis by this mechanism was investigated using a dual chamber microwell system in which supernatants of CSE-activated eosinophils were used to attract neutrophils. Supernatants of eosinophils cultured in 2.5% CSE for 18h significantly increased neutrophil migration in comparison to supernatants from eosinophils cultured in medium, alone (Fig. 6). Thus, CSE-activated eosinophils contained significant chemotactic activity for neutrophils. While IL-8 may have been responsible for this effect this was not confirmed in this study.

**Figure 6**

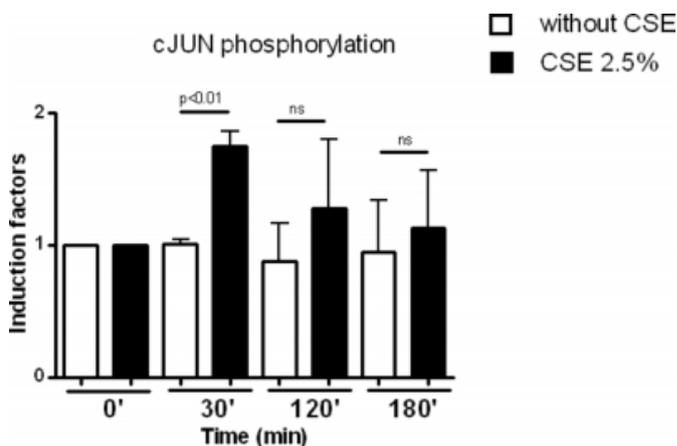
Figure 6: Supernatants from CSE-activated eosinophils enhance neutrophil chemotaxis. Neutrophils [10/ml] were incubated with supernatants of eosinophils that were incubated for 18h with or without 2.5% CSE. Neutrophil migration was evaluated as described in Materials and Methods. Results of experiments performed with two different donors are shown. Tests were performed in triplicate. Control: neutrophil migration in culture medium, alone.



In vitro exposure to CSE increases c-Jun protein levels in eosinophils. Mast cell derived tryptase induces IL-8 synthesis in human peripheral blood eosinophils [50] in a MAPK/AP-1-dependent pathway that leads to activation of c-Jun. To examine the possibility that in vitro exposure of CSE induces IL-8 release from eosinophils by a similar mechanism, eosinophils were activated for various times ranging from 0 to 360 min, and c-Jun protein levels were measured by flow cytometry after intracellular staining with specific antibodies. A transient increase in c-Jun protein expression was observed after 30 min in cells that were incubated in the presence of 2.5% CSE in comparison with cells that were incubated with medium, alone (Fig. 7). However, c-Jun levels returned to baseline after 120 min.

**Figure 7**

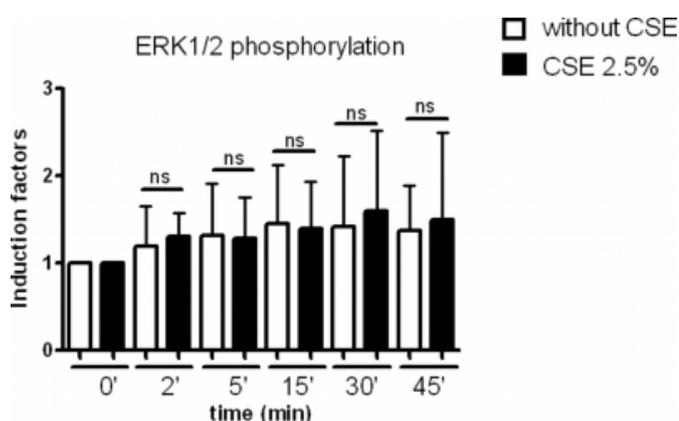
Figure 7: exposure to CSE increases cJun protein levels in eosinophils. Eosinophils [10 cells/ml] were incubated with 2.5% CSE for the indicated times and then stained intracellularly with mouse anti-cJun antibody. Intracellular staining was evaluated by flow cytometry. The results are expressed as the fold increase (induction factor), relative to baseline cJun expression. Values shown are the mean  $\pm$  SD of results from three experiments. ns = not significant



In vitro exposure to CSE does not induce ERK phosphorylation in eosinophils. Activation of transcription factors, such as c-Jun is dependent upon the phosphorylation of MAP kinases (ERK, p38 and JNK). As CSE induced transient c-Jun expression in eosinophils, we examined the possibility that CSE induced phosphorylation of ERK, p38, and JNK. Eosinophils were incubated for various times (0-180 min) with CSE (2.5%) and/or GM-CSF/TNF- $\alpha$ . Experimental groups for MAP kinases included cells incubated with: medium alone; cells exposed to CSE without other activators; cells exposed to GM-CSF + TNF- $\alpha$  without CSE; and cells exposed to GMCSF +TNF $\alpha$ and CSE. ERK1/2, p38, and JNK levels were measured by flow cytometry after intracellular staining with specific antibodies. Due to technical difficulties results were obtained only for ERK phosphorylation and are shown in Fig. 8. CSE at a concentration of 2.5% failed to induce ERK 1/2 phosphorylation in eosinophils incubated in medium, alone. Activation of eosinophils with TNF- $\alpha$  and GM-CSF induced a transient increase in ERK1/2 phosphorylation relative to non-stimulated controls (not shown). Adding 2.5% CSE had no influence on ERK1/2 phosphorylation. Thus, it was concluded that ERK1/2 does not appear to be involved in activation of c-Jun expression after exposure to CSE.

**Figure 8**

Figure 8: exposure to CSE does not induce ERK phosphorylation in eosinophils. Eosinophils were incubated for various intervals from 0-45 min with in medium without CSE (open bars) or in medium with 2.5% CSE (filled bars). Expression of pERK1/2 was assessed by flow cytometry after intracellular staining with p-ERK-specific antibody. Results shown are the mean  $\pm$  SD of triplicate experiments. ns = not significant.



## DISCUSSION

Smoking, especially in association with chronic lung conditions such as COPD, is associated with the release of many pro-inflammatory cytokines and chemokines including, TNF- $\alpha$  and IL-8 [51]. Accumulation of neutrophils in the large airways has been associated with disease severity [52]. While several studies have shown that cigarette smoke may activate lung macrophages [53], epithelial cells [54] and fibroblasts [40] to release pro-inflammatory mediators, little is known about the effects of cigarette smoke on eosinophils, one of the main effector cells in allergic inflammation in the lung, especially in the late phase of asthma. Here we show that in vitro exposure to chemicals present in cigarette smoke extract (CSE) induced the release of IL-6 and IL-8. While our study did not address the physiologic relevance and nature of the mediators involved in this effect, these findings are nevertheless intriguing. Our study shows that supernatants from CSE-activated eosinophils contained chemotactic activity for neutrophils which are major components of the late phase response in asthma and in tissue destruction in chronic lung inflammatory diseases. However, the possibility that other eosinophil-derived chemo-attractants such as leukotriene B4 (LTB4) might have been active in these supernatants cannot be excluded. Moreover, eosinophils are sources of vascular endothelial growth factor (VEGF) and other factors involved in fibrogenesis and tissue remodeling in asthma and chronic lung inflammation [8,55,56]. That CSE-exposed eosinophils

also showed ultrastructural changes suggestive of cellular activation and/or stress, supports the notion that components of cigarette smoke at low concentrations co-activate eosinophils and hence, may exacerbate the inflammatory response. Elevated levels of TNF- $\alpha$  have also been found in bronchoalveolar lavage fluid from asthmatic patients [57] and up-regulated TNF- $\alpha$  expression has been detected in alveolar macrophages [58], mast cells [59], and bronchial epithelial cells [60]. Our results show that TNF- $\alpha$  enhances the CSE-induced effect on eosinophil cytokine release. This effect is strongest together with GM-CSF, which suggests a synergistic effect. Hence, elevated levels of TNF- $\alpha$  in the pulmonary microenvironment of asthmatics might increase their sensitivity to CSE.

While at lower concentrations (<5%) CSE appeared to enhance eosinophil activation, higher concentrations and increased exposure times proved to be toxic to these cells. Addition of the eosinophil survival factor GM-CSF partially reversed this effect and rescued CSE-exposed eosinophils from death. As exposure to CSE did not result in increased detection of Annexin V, it is likely that eosinophil death (at least at CSE concentrations of 2.5% or less) was not due to apoptosis. Following activation eosinophils release survival factors such as IL-5 [61] and GM-CSF [62] that promote their own survival. Hence, it is possible that CSE also enhanced release of these survival factors or by actively inhibiting apoptosome formation as has been demonstrated for Jurkat cells [63], a cell line that undergoes apoptosis readily and which displays characteristic apoptosis markers.

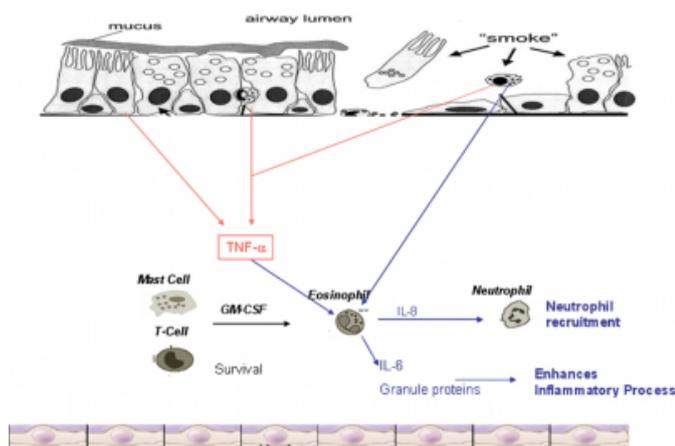
We reported earlier [64] that tryptase-activated human peripheral blood eosinophils produce IL-8 and that this production is mediated by the mitogen-activated protein kinase (MAPK)/AP-1 pathway. In this study we observed that exposure to 2.5% CSE induced the expression of c-Jun protein in eosinophils within 30 min. These results are in accordance with the previous finding that nitric oxide (NO) and reactive oxygen species (ROS), major components of cigarette smoke, cause induction of AP-1 [66,67,68]. AP-1 may be activated through the MAPK pathway via the phosphorylation of either JNK or P38 [64,67] whereas phosphorylation of ERK1/2 does not appear to be required. While technical difficulties in our study precluded clear interpretation of JNK and P38 phosphorylation, transient phosphorylation of ERK1/2 was observed. It is possible that the sensitivity of stress induced kinases to temperature changes made it difficult to detect transient phosphorylations of JNK and P38 [66], while ERK1/2 being a cytokine-induced

kinase was more resilient to temperature variations during manipulation of the cells.

The results of our preliminary studies, taken together with those of other studies, suggest that exposure to cigarette smoke may augment lung inflammation through its effects on eosinophils. How this might occur is summarized in the model shown in Fig. 9. In this model, cigarette smoke has a direct effect on eosinophils by causing the release IL-8 and IL-6 and by inducing degranulation and release of other proinflammatory mediators. As a result, neutrophils are recruited into the inflamed tissue enhancing the ongoing inflammatory process. IL-6, being a strong pro inflammatory and pro-angiogenic mediator enhances the inflammatory response. TNF- $\alpha$ , released mainly by macrophages and lung epithelial cells after exposure to CS potentiates the release of IL-8 and IL-6 by eosinophils. GM-CSF, besides being a survival factor, acts synergistically together with TNF- $\alpha$  to enhance CS-induced cytokine release from eosinophils.

### Figure 9

Figure 9: The potential role of cigarette smoke on eosinophil function in the context of chronic airway inflammation.



Although our study model is similar to those used in similar investigations to evaluate cigarette smoke effects on inflammatory cells [68], it is difficult to extrapolate its results to the in vivo situation as metabolic processes that would convert cigarette smoke constituents into more- or less-harmful metabolites could not be taken into account, nor could their concentrations. Also, experimental exposure to CSE was relatively short term; therefore, the results cannot be extrapolated to what occurs in chronic smokers who are exposed to these chemicals over many years. Nevertheless, our study identified some important potential pro-inflammatory effects of cigarette smoke through its actions on eosinophils, and points to how cigarette smoke exposure might exacerbate asthma and other chronic lung

inflammatory diseases.

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