

Cigarette Smoke Activates Human Monocytes by an Oxidant-AP-1 Signaling Pathway: Implications for Steroid Resistance

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ABSTRACT

Smoking cigarettes is a major risk factor for the development of cardiovascular and respiratory disease. Moreover, smoking-induced pathophysiology is often resistant to the anti-inflammatory effects of glucocorticoids. The nature of cigarette smoke-induced inflammation is still not defined, although neutrophil recruitment and activation seem to be consistent features. In the current study, we have used a range of approaches to demonstrate that cigarette smoke activates human monocytes and macrophages to release the CXC chemokine CXCL8 [interleukin-8 (IL-8)]. Furthermore, we show for the first time

that cigarette smoke synergizes with proinflammatory cytokines IL-1 β and tumor necrosis factor- α , and it is this interaction that confers steroid resistance to smoke-induced CXCL8 release. We go on to show that smoke-induced activation of human cells is an oxidant-mediated phenomenon acting through activator protein-1, but not nuclear factor κ B, pathway. These observations add significantly to our understanding of smoke as an inflammatory stimulus that has implications for potential the development of treatments of smoking or related disease.

Smoking-related diseases represent a worldwide leading cause of morbidity and mortality and is predicted to rank as the third most common cause of death by 2020 (Murray et al., 2001). Long-term smoking has profound pathophysiological effects on the lungs and cardiovascular systems. Cigarette smoke is the major etiological factor in the pathogenesis of chronic obstructive pulmonary disease and is associated with increased risk of lung cancer (Burns, 2003). In the cardiovascular system, epidemiological studies clearly show that smoking increases the incidence of myocardial infarction and fatal coronary artery disease (Ambrose and Barua, 2004).

Cigarette smoke evokes an inflammatory response in both humans and animal models, which is characterized by the early influx and activation of inflammatory cells together with the release of chemokines, including CXCL8 (van der Vaart et al., 2004). In addition to effects in vivo, cigarette

smoke can directly activate human cells in vitro to release mediators, including CXCL8 (Robbins et al., 1991; Mio et al., 1997; Russell et al., 2002; Numanami et al., 2003). There is no doubt that cigarette smoke is a powerful inflammatory stimulus for the lungs and the immune system. Furthermore, inflammatory cytokines, including IL-1 β and TNF α , are elevated in the lungs of smokers, suggesting that smoke may act as a coactivator of cells under these conditions.

Despite a growing body of literature describing the effects of cigarette smoke in vitro and in vivo, the signaling pathways involved in smoke-induced responses are currently still the subject of investigation. Specifically, the field has been slowed by the fact that cigarette smoke may activate (Rahman et al., 1996) and/or inhibit (Laan et al., 2004) inflammatory gene expression in in vitro models. This has meant that little or no progress has been made in the treatment of smoking-related diseases, which, unlike most inflammatory conditions, are insensitive to glucocorticoid treatment (Keatings et al., 1997). It is therefore essential for us to elucidate how smoke activates cells if we are to identify new treatment strategies for smoking-related diseases. Furthermore, the

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ABBREVIATIONS: IL, interleukin; AP-1, activator protein 1; NF- κ B, nuclear factor κ B; PBMC, peripheral blood mononuclear cell; LPS, lipopolysaccharide; SP600125, anthra[1,9-cd]pyrazol-6(2H)-one 1,9-pyrazoloanthrone; MP, *N*¹-methyl-1,9-pyrazoloanthrone; JNK, c-Jun N-terminal kinase; CSE, cigarette smoke extract; HO-1, hemeoxygenase; TNF, tumor necrosis factor; FACS, fluorescence-activated cell sorting; RPE, (R)-phycoerythrin; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; NAC, *N*-acetyl cysteine; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

pathways activated by smoke are likely to be highly relevant to a range of oxidant-dependent inflammatory conditions.

The aims of the current study are 3-fold. First, we established and characterized a cell-based model of cigarette smoke and cytokine-induced inflammation. Second, we assessed the effect of therapeutic glucocorticoids on cell activation in our model to validate its relevance to human disease. Finally, we determined the relevant contribution of the AP-1 pathway in smoke-induced activation of human monocytes, demonstrating for the first time a direct interaction of c-jun with the promoter region of CXCL8 after stimulation of cells with cigarette smoke.

Materials and Methods

Cell Culture. THP-1 human monocytes were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and cultured in RPMI 1640 medium containing 10 mM GlutaMAX and supplemented with 10% fetal calf serum, penicillin/streptomycin 100 U/ml, and nonessential amino acids. Cells were maintained in a humidified atmosphere at 37°C containing 5% CO₂. THP-1 cells were plated out onto 96-well plates at 500,000/ml in RPMI 1640 medium (0% fetal calf serum content) and were left to equilibrate for 1 h before stimulation. Unless stated otherwise, all cell culture reagents were supplied by Invitrogen (Paisley, Renfrewshire, UK).

Isolation, Morphological Examination, and FACS Analysis of Blood-Derived Monocytes and Macrophages. Human PBMC (70% lymphocytes, 30% monocytes) was isolated as described previously (Perretti et al., 1999). In brief, fresh citrated blood was layered on a Ficoll gradient (Histopaque gradient, Histopaque 1077, and Histopaque 1119) and centrifuged (400g, 20°C) for 30 min. PBMCs were collected, washed with prewarmed RPMI 1640 medium, and pelleted by centrifugation (450g, 24°C). Cells (10⁶ per ml) were plated in 96-well culture plates.

Purification of Monocytes. Monocytes were separated from contaminating lymphocytes by incubating them (37°C in a 5% CO₂) for 2 h on plastic. Nonadherent cells were removed by washing, and the purity of the culture was assessed by two techniques: initially morphologically under light microscopy, and then by flow cytometric analysis for CD14 expression and their forward and side scatter properties.

Monocyte Differentiation into Macrophages. Isolated monocytes (70%) were differentiated into macrophages using an established protocol (Rossi et al., 1998). In brief, monocytes (2 × 10⁶/ml) were cultured in six-well plates and induced to differentiate by incubation with RPMI 1640 medium containing 10% autologous serum for a period of 7 days (37°C in a 5% CO₂ atmosphere). After differentiation, macrophage phenotype was assessed by two techniques: initially morphologically under light microscopy, and then by flow cytometric analysis for CD14 expression and their forward and side scatter properties.

CD14 Staining and FACS Analysis. All staining procedures were performed at 4°C using phosphate-buffered saline containing 1.3 mM calcium chloride and 0.2% bovine serum albumin as buffer. Cells were washed and then blocked with normal human IgG (6 mg/ml) and 30 μg/ml (R)-phycoerythrin (RPE)-linked isotype control (IgG2a) monoclonal antibody (clone, DAK-G05) or 30 μg/ml anti-human CD14 monoclonal antibody (clone, TÜK4), both purchased from DakoCytomation Denmark A/S (Glostrup, Denmark) for 1 h. Cells were then washed, and FACS analysis was performed. Cell fluorescence of monocytes gated using forward and side scatter were measured in the FL2 channel using a Becton Dickinson FACScan (BD Biosciences, San Jose, CA). Mean fluorescence intensity was calculated by subtracting the mean fluorescence intensity of the DAK-G05-stained mononuclear cells from the corresponding TÜK4-stained cells.

Isolation of Human Alveolar Macrophages. Pieces of fresh lung tissue were obtained after routine, open lung surgery at the Royal Brompton Hospital, London, UK (with informed consent). The outside of the tissue was cleaned with NaCl (0.15 M). Tissue was then injected with 10 ml of NaCl (0.15 M) using a 19-gauge needle; the first few aliquots were discarded (depending on red blood cell count), and lavage was repeated until the effluent had become virtually clear. This resulted in the isolation of 10⁴ mononuclear cells/ml of NaCl. Lavage was centrifuged (300g, 10 min, 4°C), and pellets were pooled in low-protein hybridoma medium (Invitrogen) containing streptomycin sulfate (100 μg/ml), penicillin G (100 IU/ml), and L-glutamine (0.29 mg/ml). Macrophages were counted and plated at 5 × 10⁵ cells/well in 1 ml of Lymnaea peptidyl glycine α-hydroxylating monooxygenase. Cells were incubated overnight at 37°C in a humidified atmosphere containing 5.0% CO₂; nonadherent cells were removed by washing, leaving a highly enriched population (>95%, as determined by morphological characteristics and staining with CD68) of alveolar macrophages.

Preparations of Cigarette Smoke Extract and Treatment of Cells. Cells were plated at 5 × 10⁵/ml in 96-well plates. To prepare the cigarette smoke extract, Four full-strength Marlboro cigarettes (filters removed; Phillip Morris USA, Richmond, VA) were combusted through a modified 60-ml syringe apparatus, and the smoke passed through 100 ml of RPMI 1640 medium. Each cigarette yielded five draws of the syringe (to the 60-ml mark), with each individual draw taking approximately 10 s to complete. Cigarette smoke extract was then passed through a 0.25-μ filter to sterilize and remove particulate matter and was used immediately unless otherwise stated. Cells were treated between 1 and 24 h with cigarette smoke extract and/or cytokines or LPS (Sigma Chemical, Poole, Dorset, UK). After this incubation, supernatants were collected and stored at -80°C for cytokine determination. In the antioxidant experiments (using either N-acetyl cysteine or glutathione, 10–1000 μM; Sigma Chemical) and inhibitor studies [using SP600125 and N¹-methyl-1,9-pyrazoloanthrone (MP), 1–10 μM] all reagents were added 20 min before stimulation. In the experiments using glucocorticoids, cells were pretreated 2 h before stimulation, as in human monocytes. This pretreatment time was chosen using previous data demonstrating that in peripheral blood mononuclear cells and monocytic cell lines, 2 h is required for receptor activation (Paul-Clark et al., 2003). Supernatants were collected 24 h after stimulation and stored at -80°C for cytokine determination.

As described previously (Rahman et al., 1998), smoke extract "strength" was evaluated by measuring nitrite using the Griess reaction (Bishop-Bailey et al., 1997) to ensure continuity between batches. In all experiments, nitrite levels in 100% cigarette smoke extract was between 12 and 16 μM.

Determination of LPS Levels in Cigarette Smoke Extract. Smoke extract was made as above. To test LPS contamination, a Limulus assay (E-Toxate; Sigma Chemical) was performed according to the manufacturer's instructions on undiluted cigarette smoke extract.

Assessment of Cell Respiration by MTT. The effect of IL-1β (1 ng/ml), cigarette smoke extract, or the cotreatment of cigarette smoke extract and IL-1β on THP-1 cell metabolism was assessed by measuring the mitochondrial-dependent reduction of MTT (Sigma Chemical) to formazan. This was performed after all treatments.

Measurement of Cytokine Production. CXCL8 levels in cell free supernatant were determined by ELISA using commercially available matched antibody pairs following a protocol furnished by the manufacturers (R&D Systems Europe, Abingdon, UK). CXCL8 concentrations were measured at 450 nm with a reference filter at 550 nm, and results are expressed as picograms per milliliter.

Reverse Transcriptase-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction. Total RNA was isolated from THP-1 monocytes (1–24 h) after stimulation using the RNeasy Mini Kit (QIAGEN, Crawley, UK). cDNA was generated by reverse transcription using random hexamers. The cDNA (42 ng/reaction)

was used as a template in the subsequent polymerase chain reaction (PCR) analyses. Transcript levels were determined by real-time PCR (Rotor Gene 3000; Corbett Research, Sydney, Australia) using the SyberGreen PCR Master Mix Reagent Kit (Promega, Madison, WI). The sequences of CXCL8 PCR primers were: sense, 5'-GCCAACA-CAGAAATTATTGTAAAGCTT; antisense, 5'-CCTCTGCACCCAGT-TTTCCTT. Primers for GAPDH were sense, 5'-ATTCATGGCACC-GTCAAGGCT; antisense, 5'-TCAGGTCCACCACTGACACGT. Primers were used at a concentration of 0.5 μ M for real time. Cycling conditions for real-time PCR (a total of 40 cycles used) were as follows: step 1, 15 min at 95°C; step 2, 15 s at 94°C; step 3, CXCL8: 25 s at 60°C, and GAPDH: 25 s at 64°C; step 4, 22 s at 72°C. Data from the reaction were collected and analyzed by the complementary computer software (Corbett Research). Relative quantifications of gene expression were calculated using standard curves and were normalized to GAPDH.

Measurement of Hemeoxygenase-1 Expression and Nrf2. Hemeoxygenase-1 (HO-1) expression in THP-1 cells was measured as we have described previously (Stanford et al., 2003). In brief, cells were plated into six-well culture plates and were treated with cigarette smoke or IL-1 β or IL-1 β plus smoke or LPS for 24 h for HO-1 and 12 h for Nrf2 (Pi et al., 2003). The medium was removed, and cells were washed twice with ice-cold phosphate-buffered saline. For HO-1, cells were lysed using 10 mM HEPES containing 3 mM MgCl₂, 40 mM KCl, 5% glycerol, 0.3% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. For Nrf-2, nuclear and cytosolic proteins were extracted using a recommended nuclear extraction kit (ActiveMotif, Rixensart, Belgium). Protein concentration in nuclear, cytosol, and whole-cell preparations were measured using the Bradford assay (Bishop-Bailey et al., 1997). Samples were separated by gel electrophoresis on 12% SDS-polyacrylamide gels; after transfer onto nitrocellulose membranes, HO-1 and Nrf-2 was detected using specific polyclonal rabbit antibodies (1:1000; Calbiochem, MerckBiosciences, Cambridge, UK; and 1:500, H-300; Santa Cruz Biotechnology, Santa Cruz, CA), and the signal was amplified with a goat anti-rabbit IgG-horseradish peroxidase (1:1000; DakoCytomation). Blots were visualized and captured using a GDS 8000 system attached to an Epi Chemi II darkroom after incubation with an extended duration chemiluminescence substrate (Biowest, UVP, Cambridge, UK). After the blots were stripped using Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL), the blots were probed with β -actin (1:10000; Abcam, Cambridge, UK) for cytosolic HO-1 and Laminin B (1:750; Santa Cruz Biotechnology) for nuclear Nrf-2, which was used to normalize for sample loading.

Transcription Factor Assay. Active nuclear AP-1 (c-Jun, c-Fos, and Fos B) and NF- κ B (p50, p65, RelB, and p100) levels were measured using a commercially available ELISA-based transcription factor assay (ActiveMotif) in which the relevant response elements was fixed to the base of the well. Target proteins are immobilized by binding with appropriate response element in the well and detected using selective antibodies. Duplicate wells were stimulated with either cigarette smoke extract (10%) or IL-1 β (1 ng/ml) or a combination of the two stimuli for 1 h. For NF- κ B, wells were pooled, and cells were lysed using the lysis buffer supplied by manufacturer (ActiveMotif). For AP-1, duplicate wells were pooled and subjected to nuclear fraction extraction using the recommended kit (ActiveMotif). Protein content was assessed by the Bradford assay. Assays were performed as instructed by the manufacturer, using 10 μ g of total protein. Results are expressed in optical density units per microgram of protein.

Chromatin Immunoprecipitation. THP-1 cells were treated with cigarette smoke extract (10%), IL-1 (1 ng/ml), or a combination of both for 0.5 and 2 h. Protein-DNA complexes were fixed by formaldehyde (1% final concentration) and treated as described previously (Hecht and Grunstein, 1999). Cells were resuspended in 200 μ l of SDS lysis buffer (50 mM Tris, pH 8.1, 1% SDS, 5 mM EDTA, and complete proteinase inhibitor mixture) and subjected to three 10-s pulses of sonication on ice. Sonicated samples were centrifuged to

spin down cell debris, and the soluble chromatin solution was pre-cleared using sonicated salmon sperm DNA-agarose slurry (Upstate Biotechnology, Buckingham, UK) and then immunoprecipitated using a c-jun antibody (rabbit anti-c-jun 1:50; ActiveMotif) or rabbit IgG (DakoCytomation). Protein-bound immunoprecipitated DNA was washed with LiCl wash buffer and Tris-EDTA buffer, and immune complexes were eluted by adding elution buffer (1% SDS and 0.1 M NaHCO₃). The elution was treated successively for 4 h at 65°C in 200 mM NaCl, 1% SDS to reverse cross-links, and incubated for 1 h at 45°C with 70 μ g/ml proteinase K (Sigma Chemical). DNA was extracted with phenol/chloroform; precipitated with ethanol, 0.3 M NaHCOOH, and 20 μ g of glycogen; and resuspended in 50 μ l of Tris-EDTA buffer. Quantitative PCR was performed with 10 μ l of DNA sample and 30 cycles. Primer pairs to amplify the AP-1 element within the CXCL8 promoter were as follows: forward, 5'-AGGAAGT-GTGATGACTACGGTT-3'; and reverse, 5'-GAGGAAATTCAC-GATTTGC-3'. As a control, duplicate samples were amplified using primer pairs for Oct-1 element within the CXCL8 promoter and were as follows: forward, 5'-CCAAATTGTGGAGCTTCAGT-3'; and reverse, 5'-TCAAATACGGAGTATGACGAAA-3'. PCR products were resolved by 3% agarose gel and visualized with ethidium bromide.

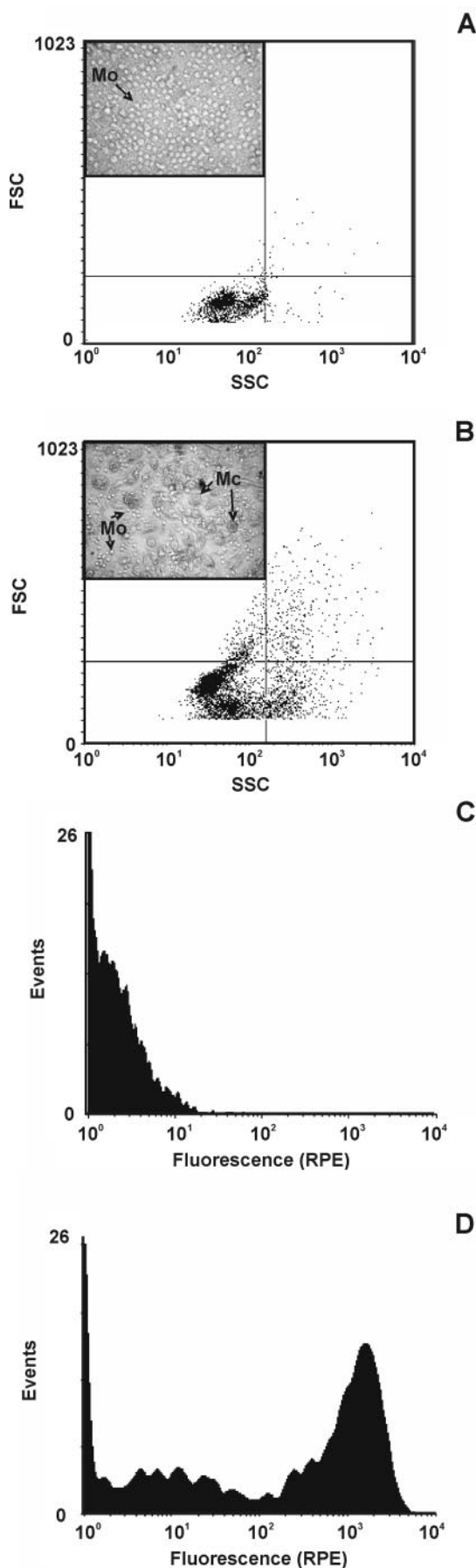
Immunocytochemistry. THP-1 cells (2×10^6) were cultured as above in 12-well plates in the presence of cigarette smoke extract (CSE), IL-1 β , or a combination of both or vehicle control (RPMI 1640 medium) for either 0.5 or 2 h. Aliquots (10^5 cells) were removed, cytospun (200g for 4 min) onto glass slides, air-dried, and then fixed in ice-cold acetone-methanol [50:50 (v/v)] at -20°C for 10 min. Cells were stained for c-jun (rabbit anti-c-jun 1:50; Calbiochem) using a previously described protocol for intracellular fluorescence staining (Ito et al., 2000). Stained cells were observed under oil immersion objective lens by confocal microscopy (with a Leica confocal microscope, equipped with a 488- and 514-nm dual-band argon laser), and images were collected using TCSNT software (Leica, Wetzlar, Germany).

Statistical Analysis. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by a Dunnett's post test or one-sample *t* test for normally distributed data. All data shown are mean \pm S.E.M.

Results

Quantification and Identification of Human Blood Monocytes and Macrophages

Monocytes were isolated from whole human blood using a Ficoll method, and their purity was tested using light microscopy and CD14 expression. After cells were allowed to adhere for 2 h, PBMCs were identified using the monocyte/macrophage marker, CD14, and immunofluorescence was quantified by FACS analysis. Monocytes were gated using their forward and side scatter properties. This revealed that $70 \pm 8\%$ ($n = 3$; Fig. 1A) of the PBMCs stained positively for CD14, with a significant increase of 582 mean fluorescent units (Fig. 1D) compared with the IgG2a isotype control (Fig. 1C). These findings were confirmed using light microscopy (Fig. 1A, inset) and established that the majority of the treated cells were from a monocyte/macrophage lineage. Matched preparations of monocytes from individual donors were further treated with autologous serum for 7 days, and the purity of the culture was assessed as before. FACS analysis (Fig. 1B; CD14) and light microscopy (Fig. 1B, inset) revealed that after 7 days, $56 \pm 11\%$ ($n = 3$) of the monocytes initially isolated had differentiated into macrophages. This confirms that the major cells used in further experiments were macrophages.



Temporal Stability of the Activate Components of CSE

Initial experiments using smoke revealed that the active component of smoke responsible for the induction of CXCL8 was subject to decay. It is important to note that cigarette smoke extract (10%) could only optimally induce CXCL8 release from THP-1 cells for up to 1 h after preparation (1167 ± 211 at time 0 to 1044 ± 307 pg/ml at 1 h). After a period of 2 h on the bench, the ability of 10% smoke to induce CXCL8 release had decreased by 47% to 553 ± 163 pg/ml. The ability of cigarette smoke extract to induce CXCL8 from cells continued to decrease slowly over a 24-h period of "bench decay", such that only 18% (214 ± 53 pg/ml) of its original activity was retained. To test whether the induction of CXCL8 caused by smoke was related to LPS contamination, endotoxin levels were measured in undiluted smoke using the Limulus assay. Undiluted CSE prepared on 3 different days contained no detectable levels of LPS (less than 100 pg/ml).

CSE Induces the Release of CXCL8 from Human Primary Blood Monocytes, Blood-Derived Macrophages, and Lung Macrophages: Interactions with IL-1 β

We tested the hypothesis that cigarette smoke extract alone and in combination with IL-1 β enhances the release of the inflammatory chemokine CXCL8 from primary human monocytes and macrophages. Preliminary experiments revealed that either smoke (1–10%) or IL-1 β (1 ng/ml) caused a dose-dependent increase in CXCL8 levels in all three types of primary human monocyte/macrophages, with no detrimental effect on cell metabolism in all cells. Maximal stimulation for CXCL8 was obtained with 2.5% cigarette smoke extract and 1 ng/ml IL-1 β . The combination of cigarette smoke extract (2.5%) and IL-1 β (1 ng/ml) caused a synergistic release of CXCL8 in both human PBMCs (Fig. 2A) and primary lung macrophages (Fig. 2C) compared with IL-1 β or cigarette smoke extract alone. In contrast, macrophages differentiated with human autologous serum for 7 days released a relatively large amount of CXCL8 in response to smoke, with IL-1 β producing a relatively small effect. Furthermore, a maximal stimulation of CXCL8 release from these cells was obtained with smoke alone, with little to no additive or synergistic effect observed when IL-1 β was added simultaneously (Fig. 2B).

Once we had established that cigarette smoke extract activates primary cultures of human monocytes and macrophages, it was important to address the intracellular signaling pathways involved in this response. However, because of the difficulties with working with primary cultures and signaling pathways, we used the premonocytic cell line THP-1 for subsequent protocols.

Fig. 1. Characterization of cell types by FACS analysis and CD14 expression in adherent PBMCs and blood-derived macrophages. PBMCs were adhered to culture plastic for 2 h, washed, and then incubated with autologous serum for 7 days; cells were then assessed morphologically for shape change and the expression of CD14 by FACS. Monocytes are at the bottom of the lower left side quadrant of A and B. A, FACS analysis of PBMCs that were allowed to adhere for 2 h. Inset, morphology by light microscopy of the same cells. B, PBMCs that have been incubated with autologous serum for 7 days. Inset, morphology by light microscopy of the same cells. C, cells that have been treated with IgG2a isotype (clone, DAK-G05) control linked to RPE. and D, cells that have adhered to plastic for 2 h and were treated with a monoclonal anti-human CD14 antibody (clone, TÜK4) linked to RPE. Diagrams are representative of three different subjects carried out over 3 different experimental days.

CSE Induces the Release of CXCL8 Protein and Increased mRNA for CXCL8 in THP-1 Cells: Comparisons with IL-1 β

Similarly to observations made in primary cells, CSE activated THP-1 cells to release CXCL8 over a 24-h experimental

period. Specifically, increasing concentrations of cigarette smoke extract (1–10%) caused a significant concentration-dependent increase in CXCL8 levels ($p \leq 0.05$; maximum levels of 1364 ± 80 versus 52 ± 25 pg/ml at baseline). Concentrations of cigarette smoke extract 20% and greater abrogated the increase in CXCL8 levels from THP-1 cells; however, this dramatic decrease was not associated with a significant reduction in cellular metabolism, as assessed by MTT assay. Under control culture conditions, THP-1 cells released low levels (82 ± 4 pg/ml) of IL-8 over the 24-h experimental period. However, when cells were treated with increasing concentrations of CSE, IL-8 release was induced in a bell-shaped manner (Fig. 3). Inhibition of CXCL8 levels at 20% CSE and greater could not be accounted for by an inhibition of cellular metabolism. In repeated experiments ($n = 14$), a 10% smoke concentration resulted in the most consistent increase in CXCL8 levels; therefore, this was used in all subsequent experiments.

The temporal nature of smoke-induced CXCL8 release was investigated. Cigarette smoke extract induced significant increases in CXCL8 protein release after 8- and 24-h stimulation (Fig. 4A; $p \leq 0.05$). Likewise, when cells were stimulated with IL-1 β , CXCL8 release was increased at the 8- to 24-h time points (Fig. 4B; $p \leq 0.05$). As with the primary lung alveoli macrophages and blood-derived monocytes, a combination of cigarette smoke extract (10%) and IL-1 β (1 ng/ml) resulted in a synergistic effect on CXCL8 release (Fig. 4C). The synergy achieved between smoke and IL-1 β was not exclusive to this cytokine because similar results on CXCL8 release (measured at 24 h) were obtained with TNF α (basal, 36 ± 11 pg/ml; TNF α , 61 ± 3 pg/ml; TNF α and cigarette smoke extract, 3917 ± 712 pg/ml). To determine whether the ability of smoke to release CXCL8 was regulated at the level of new protein synthesis, experiments were performed using the inhibitor cycloheximide. Cycloheximide (0.3–10 μ M) caused a concentration-dependent inhibition of CXCL8 release from THP-1 cells stimulated with smoke, IL-1 β , or the combination of smoke plus IL-1 β .

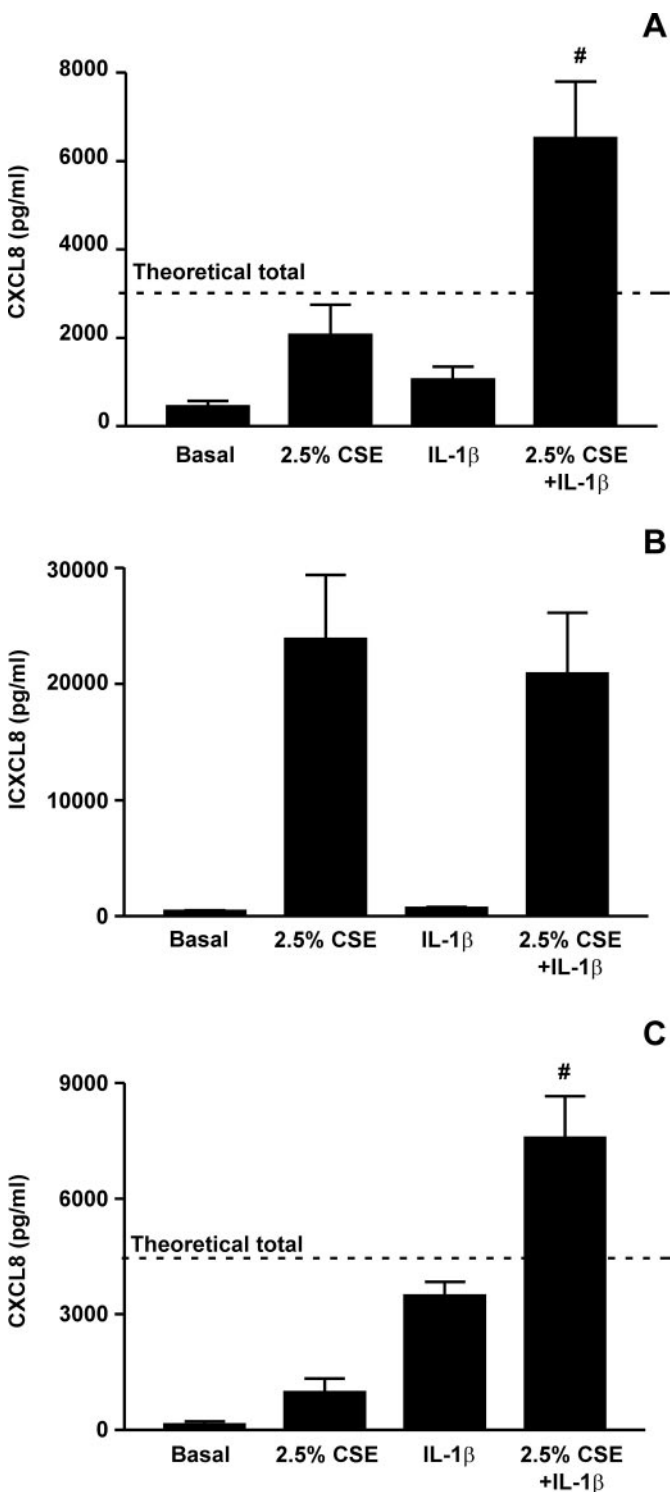


Fig. 2. Characterization of CSE-induced CXCL8 release by cultures of human primary monocytes, blood-derived macrophages, and lung tissue-derived macrophages. The effects of CSE (2.5%) or IL-1 β (1 ng/ml) or a combination of both were assessed on CXCL8 release by human peripheral blood monocytes (A), human autologous serum-derived blood macrophages (B), or human lung tissue-derived macrophages (C). The data are the mean \pm S.E.M. for cells from $n = 3$ separate donors. #, synergy.

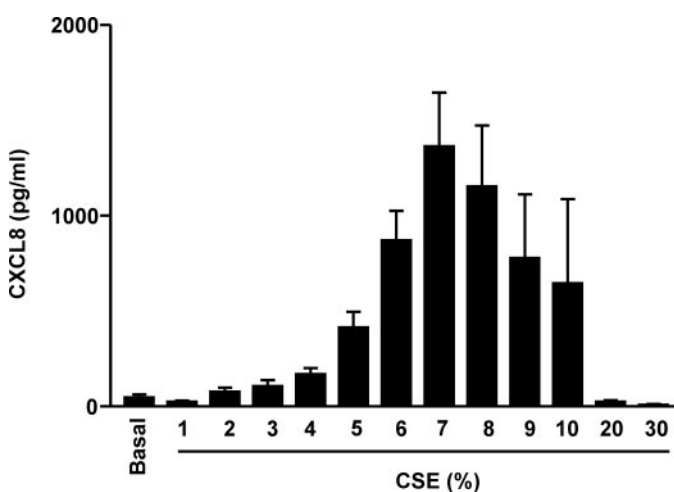


Fig. 3. The effect of increasing concentrations of CSE on IL-8 release from THP-1 cells. Cells were stimulated with CSE concentrations from 1 to 30% for 24 h. Cell-free supernatant was assessed for IL-8 by ELISA. CSE induced CXCL8 release from THP-1 monocytes in a bell-shaped manner. At CSE concentrations lower than 5%, no increase (over basal) in IL-8 release was observed. At concentrations between 5 and 10% CSE, IL-8 release was induced. At 20% CSE and greater, IL-8 release was inhibited; $n = 9$ from 3 experimental days.

Next, we investigated the role of gene activation in smoke-induced CXCL8. Likewise, to observations made with authentic protein, cigarette smoke extract or IL-1 β induced significant increases in CXCL8 mRNA levels. Once again, the smoke and IL-1 β acted synergistically to increase levels of CXCL8 mRNA (Fig. 5).

CXCL8 Release Induced by CSE and IL-1 β Is Functionally Resistant to Inhibition by Clinically Relevant Glucocorticoids

Smoking-related inflammation in humans is notoriously resistant to inhibition by glucocorticoids. Budesonide and prednisolone are currently used therapeutically in the treatment of responsive inflammatory conditions in humans. To assess how well our “model” of smoke-induced cell activation is to the clinical setting, we investigated the effects of budesonide and prednisolone on smoke-induced CXCL8 release.

Both budesonide and prednisolone caused concentration-dependent reductions in the release of CXCL8 induced by

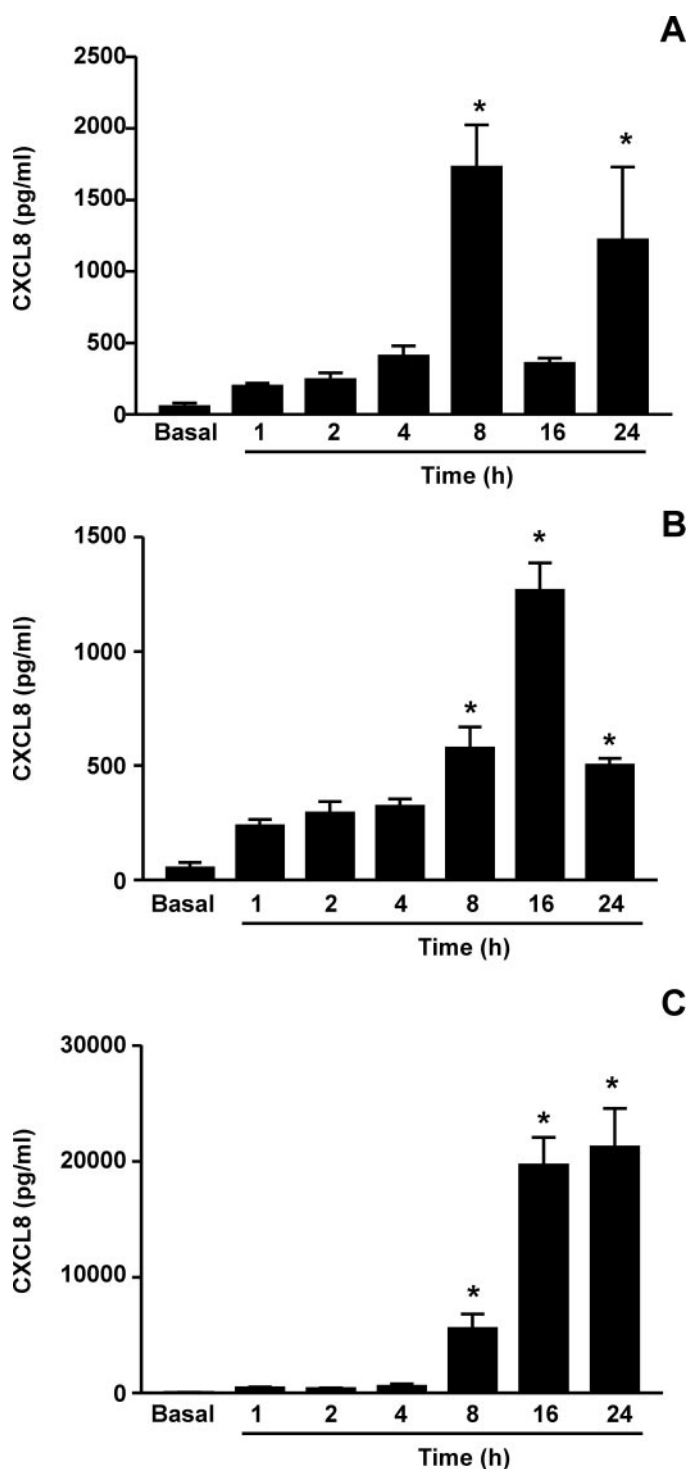


Fig. 4. Time course of CXCL8 release from THP-1 cells. THP-1 cells were treated over a 24-h period with either 10% CSE (A), IL-1 β (1 ng/ml) (B), or a combination of 10% CSE and IL-1 β (1 ng/ml) (C). The data represent the mean \pm S.E.M. for an $n = 9$ from 3 experimental days. *, $p < 0.01$ as determined by ANOVA followed by Dunnett's post test.

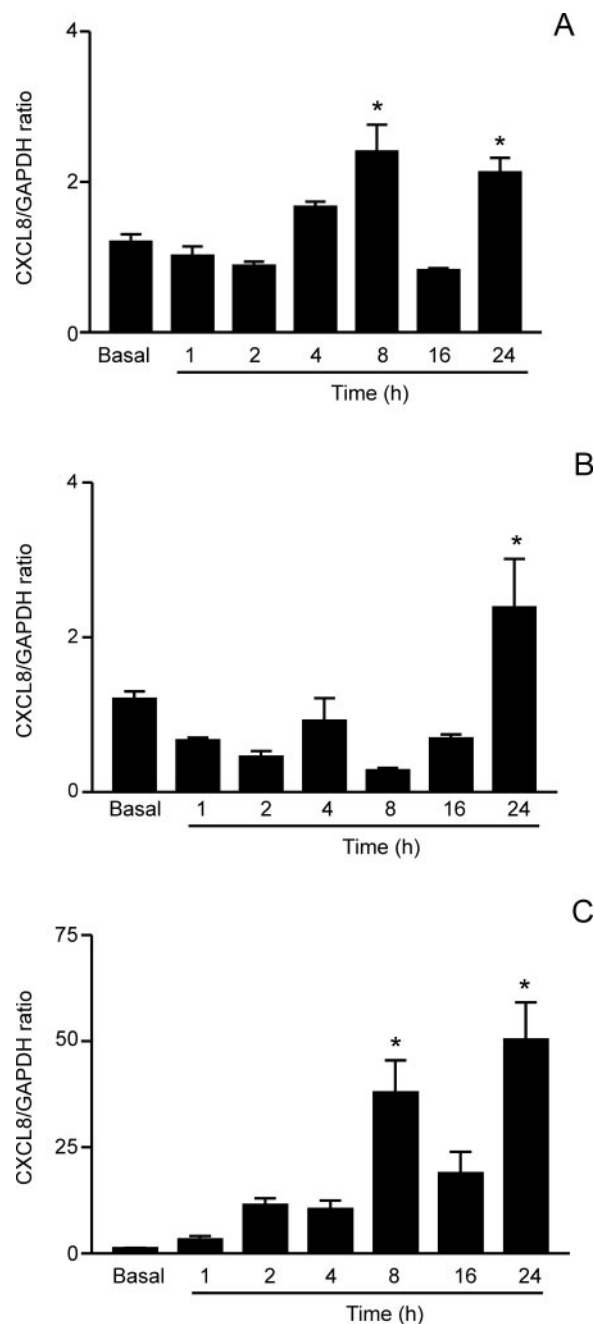


Fig. 5. Time course of CXCL8 mRNA from THP-1 cells. THP-1 cells were treated over a 24-h period with either 10% CSE (A), IL-1 β (1 ng/ml) (B), or a combination of 10% CSE and IL-1 β (1 ng/ml) (C). The data represent the mean \pm S.E.M. for an $n = 9$ from 3 experimental days. *, $p < 0.01$ as determined by ANOVA followed by Dunnett's post test.

cigarette smoke extract (Fig. 6A). It is interesting to note, however, that neither glucocorticoid had any effect on the CXCL8 release induced by IL-1 β (Fig. 6B) or by the combination of IL-1 β plus cigarette smoke extract (Fig. 6C).

Characterization of the Intracellular Signaling Pathways Activated by Cigarette Smoke Extract in the Release of CXCL8

Oxidative Stress. Cigarette smoke consists of more than 10^{16} free radicals per puff (Church and Pryor, 1985). It is

therefore predictable that smoke induces oxidative stress within cells. To determine this, we measured the ability of smoke to induced the intracellular oxidant sensor protein HO-1 (Favatier and Polla, 2001) and the redox-sensitive transcription factor Nrf-2 (Pi et al., 2003). Under basal culture conditions, THP-1 cells contained undetectable levels of HO-1. However, after stimulation with cigarette smoke extract alone (5–20%) or in combination with IL-1 β , a clear induction of HO-1 was observed (Fig. 7A). With Nrf-2 after a 12-h incubation with CSE or IL-1, there was no increase in nuclear expression of this protein. However, when CSE (5–20%) was coincubated with IL-1 β , a translocation of Nrf-2 from cytosol to the nucleus was observed (Fig. 7B).

Next, we investigated whether the oxidative stress caused by smoke contributed to the induction of CXCL8 release under these conditions. The antioxidant *N*-acetyl cysteine (NAC; 0.01–1 mM) caused a concentration-dependent protection of THP-1 cells from the effects of CSE (Fig. 7B). In contrast, NAC had no effect on IL-1 β -induced CXCL8 release (Fig. 7C). It is of interest that the release of CXCL8 induced by cigarette smoke extract plus IL-1 β was, like CSE, inhibited by NAC (Fig. 7D). In light of the finding that oxidative stress plays an important regulatory role in the activation of CXCL8, oxidative-sensitive signaling pathways were investigated as possible mechanisms in the effects of cigarette smoke.

c-Jun N-Terminal Kinase. c-Jun N-terminal kinases (JNKs) can be activated by oxidative stress and are therefore likely to be involved in smoke-induced activation of cells. Similar to observations made with NAC and glutathione (data not shown), pharmacological inhibition of JNK using SP600125 inhibited CXCL8 release induced by smoke alone (Fig. 8A) and in combination with IL-1 β (Fig. 8E) but not IL-1 β (Fig. 8C) at a selective concentration (i.e., $<10 \mu\text{M}$). The control compound MP had no effect on any stimuli at all concentrations tested (1–10 μM ; Fig. 8, B, D, and F). Therefore, these experiments suggest that JNKs mediate smoke-induced CXCL8 release from monocytes/macrophages.

NF- κ B. When cells were stimulated with IL-1 β , active nuclear p65 levels were maximally elevated at 1 h after stimulation (Fig. 9A). In contrast, NF- κ B activity was not increased in cells stimulated with cigarette smoke extract (10%; Fig. 9A). Costimulation with cigarette smoke extract and IL-1 β produced levels of p65 activation similar to those seen in cells stimulated with IL-1 β alone (Fig. 9A). No effect was observed of any treatment on the active levels of either p52 or RelB (data not shown). Inhibition of THP-1 cells with a NF- κ B kinase 2 inhibitor resulted in a marked inhibition of CXCL8 levels by all stimuli used (data not shown).

AP-1. JNK has been shown to phosphorylate serine and threonine residues in the N-terminal regions of members of the jun family of proteins (Pulverer et al., 1991; Smeal et al., 1991). This is consistent with the data obtained in this study in which treatment of cells with cigarette smoke extract induced the release of active phospho-c-jun to the nucleus, with no change in Fos-B or C-Fos (data not shown) and pronounced c-jun binding to human CXCL8 gene (Fig. 9B). Likewise, IL-1 β increased c-jun nuclear association and direct binding of c-jun to the CXCL8 promoter as determined by chromatin immunoprecipitation assay (Fig. 9B). There was a distinct additive effect on c-jun binding to CXCL8 gene when cells were costimulated with smoke plus IL-1 β (Fig.

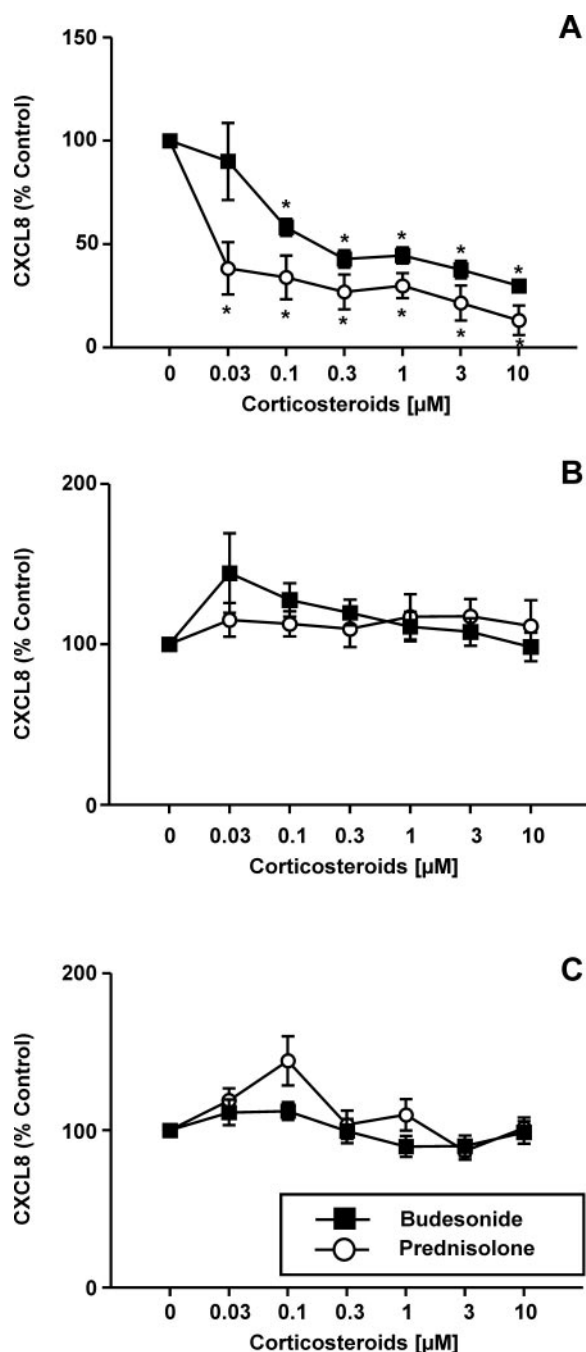


Fig. 6. Glucocorticoids were unable to inhibit CXCL8 release from THP-1 cells after stimulation with a combination of CSE or IL-1 β . The effects of budesonide or prednisolone (both 0.3–10 μM) were assessed on CXCL8 release from THP-1 cells stimulated with either 10% CSE (A), IL-1 β (1 ng/ml) (B), or a combination of 10% CSE and IL-1 β (1 ng/ml) (C). The data represent the mean \pm S.E.M. for an $n = 9$ from 3 experimental days. *, $p < 0.01$ as determined by ANOVA followed by Dunnett's post test.

9B). Samples immunoprecipitated with c-jun showed no amplification products when real-time PCR was used to detect Oct-1 or immunoprecipitated with rabbit IgG and detected using primers for AP-1 binding site, demonstrating selectivity for c-jun and AP-1 in the human CXCL8 promoter region using the antibodies and primers described above.

Discussion

The nature of the inflammatory response induced by cigarette smoke is currently the subject of scientific investigation. Here, we make several important advancements in the understanding of smoke as an inflammogen. First, we demonstrate that cigarette smoke produces a bell-shaped induction of CXCL8 expression. Second, we demonstrate that cigarette smoke can synergize with the inflammatory cytokines

TNF α and IL-1 β to cause a "superinduction" of CXCL8 release, which is at the level of new gene transcription. Third, we show that when primed with IL-1 β , cigarette smoke-induced CXCL8 release is resistant to inhibition by glucocorticoids. This observation is important clinically because smoking-related inflammation, such as that seen in chronic obstructive pulmonary disease, is resistant to treatment with steroids (Keatings et al., 1997). Finally, for the first time, we provide evidence that cigarette smoke induces c-jun binding to an AP-1 site in the promoter region of the CXCL8 gene and that this event, and not NF- κ B activation, is involved in the synergistic release of mature CXCL8 in monocytes and macrophages treated with smoke and IL-1 β .

Cigarette smoke has been shown to either activate (Rahman et al., 1996) or inhibit the activation (Ouyang et al., 2000; Witherden et al., 2004) of isolated cells in culture.

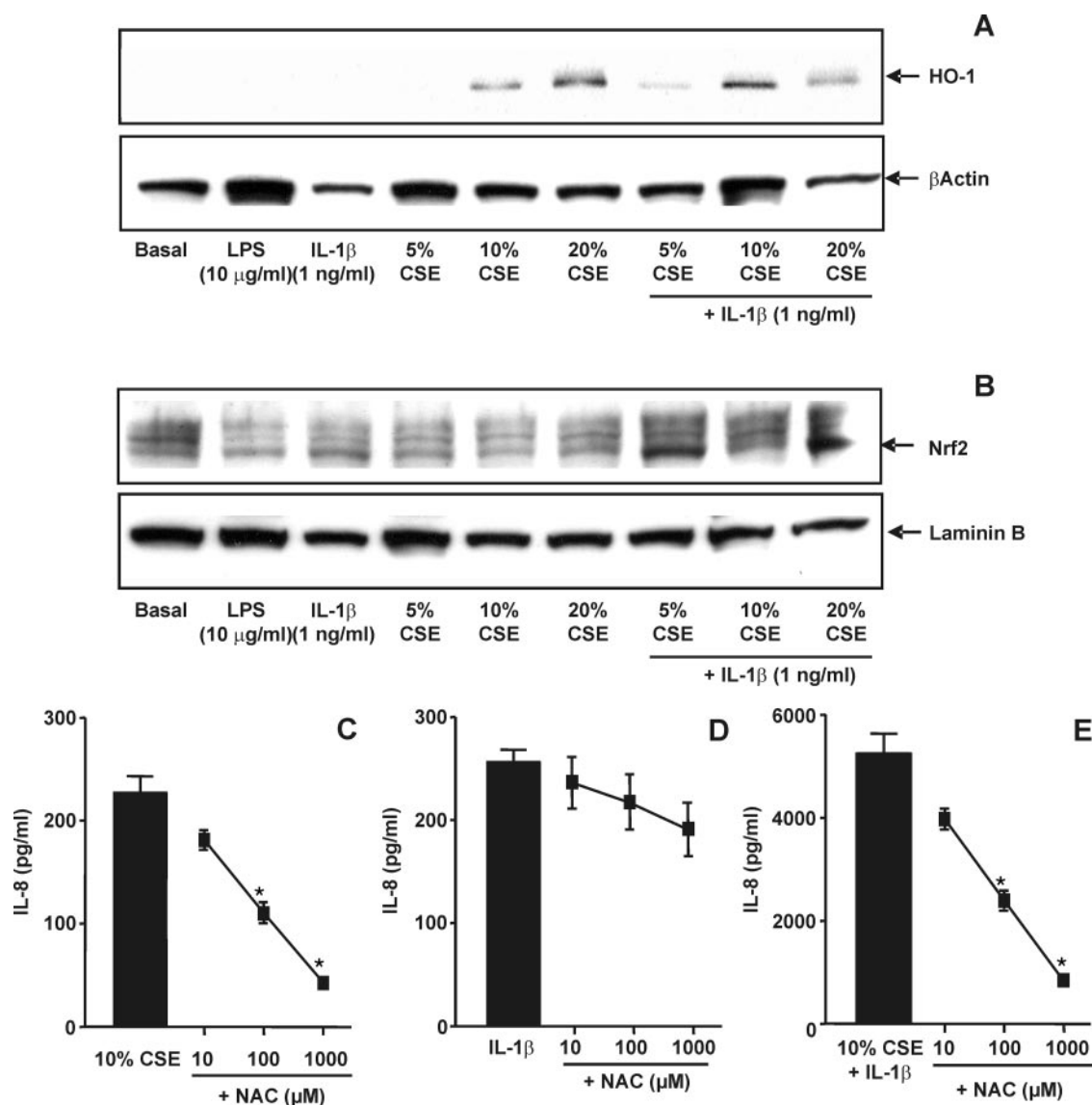


Fig. 7. Assessment of oxidative stress in THP-1 cells induced by CSE and IL-1 β . The effect of LPS (10 μ g/ml), IL-1 β (1 ng/ml), CSE (5–20%), and a combination of CSE (5–20%) and IL-1 β (1 ng/ml) was assessed on the expression of the oxidative-sensitive protein HO-1 with β -actin protein loading control (A) and nuclear expression of the redox-sensitive transcription factor Nrf-2 with Laminin B as nuclear protein loading control (B). Because the oxidative stimulus CSE in combination with IL-1 β induced HO-1 expression, the effects of the antioxidant NAC was assessed on CXCL8 release from THP-1 cells treated with either 10% CSE alone (C), IL-1 β (1 ng/ml) (D), or a combination of 10% CSE and IL-1 β (1 ng/ml) (E). Note the differences in scales between parts C, D, and E. The data represent the mean \pm S.E.M. for an $n = 9$ from 3 experimental days. *, $p < 0.01$ as determined by ANOVA followed by Dunnett's post test.

However, in the current study, we consolidate these apparent contradictory results in the literature by showing that smoke can evoke both responses and that it is an effective concentration of smoke that dictates which overall effect will be seen. Our observations showing that smoke stimulates CXCL8 release are particularly robust, having demonstrated this phenomenon in primary monocytes, monocyte-derived macrophages, lung macrophages, and the premonocytic cell line THP-1. We went on to study the mechanisms involved in smoke-induced activation of CXCL8 release, rather than inhibition, because *in vivo*, the effect of smoke is proinflammatory (Pesci et al., 1998). It is interesting that there is a

biphasic response in the induction of CXCL8 gene expression in cells stimulated with smoke or with smoke plus IL-1 β with an initial peak at 8 h, followed by a decrease at 16 h, and a second peak at 24 h. In contrast, when cells were treated with IL-1 β alone, increased levels of CXCL8 mRNA displayed one peak at 16 h. This results in a two-stage release of mature CXCL8 in monocytes that correspond to these peaks. This suggests, not surprisingly, that there may be two distinct pathways involved in superinduction of CXCL8 by the combination of IL-1 β and smoke.

In previous reports, the effects of cigarette smoke on inflammatory gene induction have been compared with those of

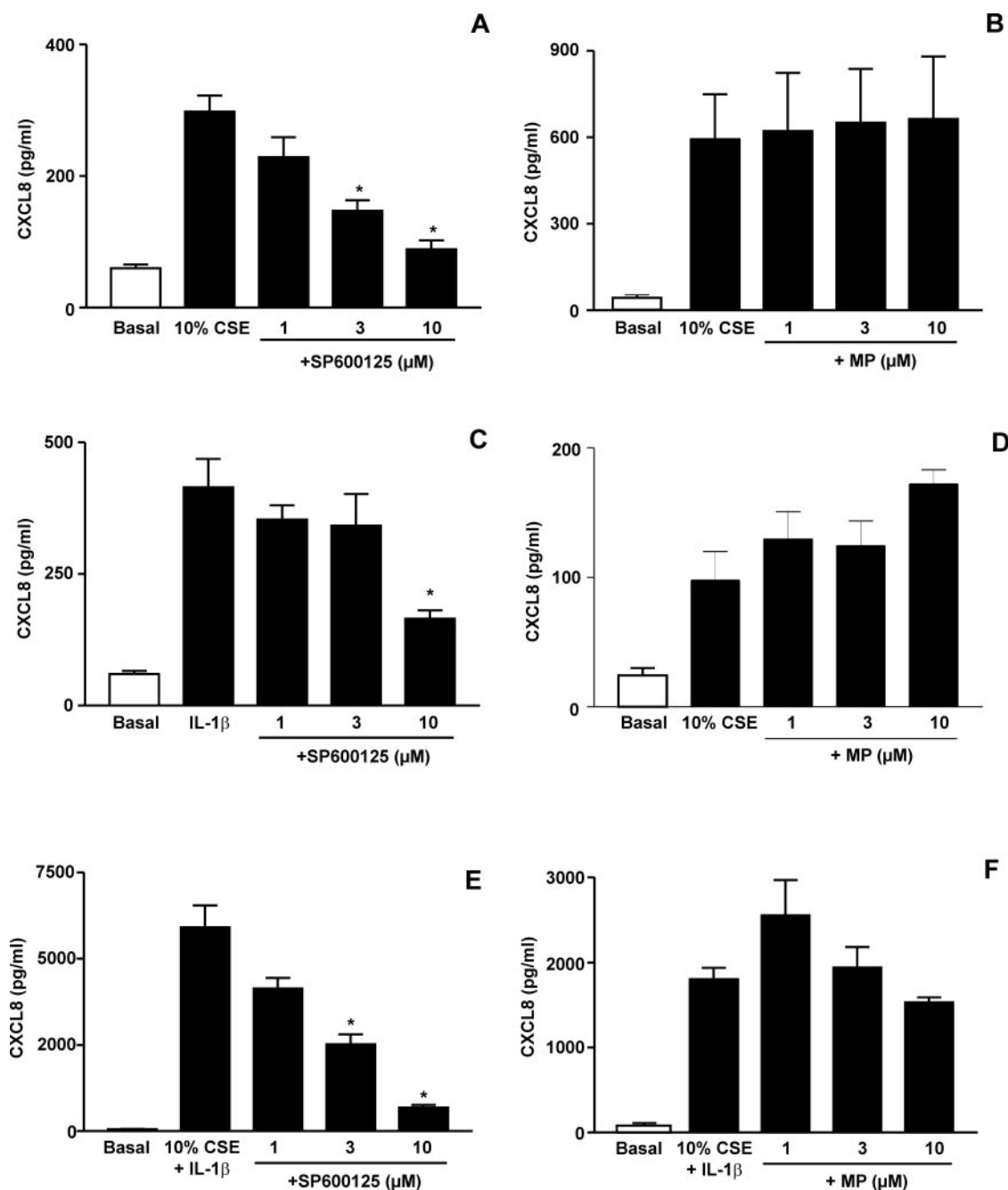


Fig. 8. Concentration-dependent inhibition of CXCL8 by the JNK kinase inhibitor SP600125. The effects of SP600125 (1–10 μ M) and control compound MP (1–10 μ M) added 20 min before activation were assessed on CXCL8 release after 24 h from THP-1 cells stimulated with either 10% CSE (A and B), IL-1 β (1 ng/ml) (C and D), or a combination of 10% CSE and IL-1 β (1 ng/ml) (E and F). The data represent the mean \pm S.E.M. for an $n = 9$ from 3 experimental days. *, $p < 0.01$ as determined by ANOVA followed by Dunnett's post test.

cytokines. However, this report is the first to study the potential interactions between smoke and cytokines in the activation of cells, in this case, to release CXCL8. We suggest that this is a particularly important area of study because in smokers, at areas of inflammation, cells are inevitably going to be exposed to both cytokines (including IL-1 β) and smoke (Pesci et al., 1998). We found that when monocytes or lung macrophages were costimulated with smoke and IL-1 β , there is a profound synergy between the two stimuli, resulting in

the release of large amounts of CXCL8. To our knowledge, this phenomenon has not been reported previously.

Extending these observations, we noted that when cells were costimulated with IL-1 β plus smoke, the resultant release of CXCL8 was completely resistant to inhibition by glucocorticoids. In contrast, when cells were stimulated with smoke alone, the release of CXCL8 was strongly inhibited by glucocorticoids. These observations have direct clinical relevance, as mentioned above; smoking-related inflammation is often resistant to the therapeutic benefits of glucocorticoids (Culpitt et al., 2003). We suggest, therefore, that where in vitro assays are used to identify new pharmaceutical preparations for the treatment of smoking-related disease, cells should be stimulated with the combination of smoke plus IL-1 β .

It is not currently clear how cytokine priming confers steroid resistance to a smoke-induced inflammatory response. However, given our observations that IL-1 β , but not cigarette smoke, induces the activation of NF- κ B (see below) in our cells, it is tempting to speculate that steroid resistance here is mediated by the expression of NF- κ B inducing the well-described phenomenon of NF- κ B transrepression of glucocorticoid receptor α (McKay and Cidlowski, 1999). The pathways involved in this are currently the subject of continued research.

The induction of genes during inflammation is regulated by well-described transcription factor pathways. Perhaps the most ubiquitous of these are the NF- κ B (Bonizzi and Karin, 2004) and AP-1 pathways (Shaulian and Karin, 2002). The current literature describing the effects of smoke on AP-1 and NF- κ B is somewhat contradictory. For example, smoke has been shown to activate (Shishodia et al., 2003), inhibit the activation (Favatier and Polla, 2001), or not affect NF- κ B (Moodie et al., 2004). Likewise, smoke may activate (Gensch et al., 2004), inhibit the activation (Laan et al., 2004), or not affect AP-1-mediated signaling (Favatier and Polla, 2001). As mentioned above, we suggest that the lack of clarity in the effects of smoke on these pathways is probably related to the active concentration used in individual studies, in which activation or inhibition of target genes has been described equally. In the current study, we initially identified the involvement of oxidant stress in smoke-induced CXCL8 release. Oxidant stress is increasingly recognized as a trigger for the activation of either NF- κ B or AP-1 (Maziere et al., 1999). However, our data show that in our cells, smoke does not cause a substantial activation of NF- κ B. The effects here are in contrast to those of IL-1 β , which predictably increased NF- κ B activity in our cells. However, the NF- κ B kinase 2 inhibitor substantially reduced CXCL8 levels after activation with all stimuli. These observations effectively rule out NF- κ B in the superinduction of CXCL8 in monocytes and macrophages by smoke and IL-1 β in our system. However, there is some requirement for tonic levels of NF- κ B in the activation and release of CXCL8 from human macrophages.

Reactive oxidative species has been demonstrated to up-regulate JNK, which in turn activates Jun family members (Pulverer et al., 1991; Smeal et al., 1991). In the current study, we found that the JNK inhibitor SP600125 blocked smoke-induced CXCL8 release. This observation clearly suggests a role for AP-1 in smoke-induced CXCL8 release. This notion is consistent with recent observations using lung epithelial cells stimulated with smoke, in which the release of

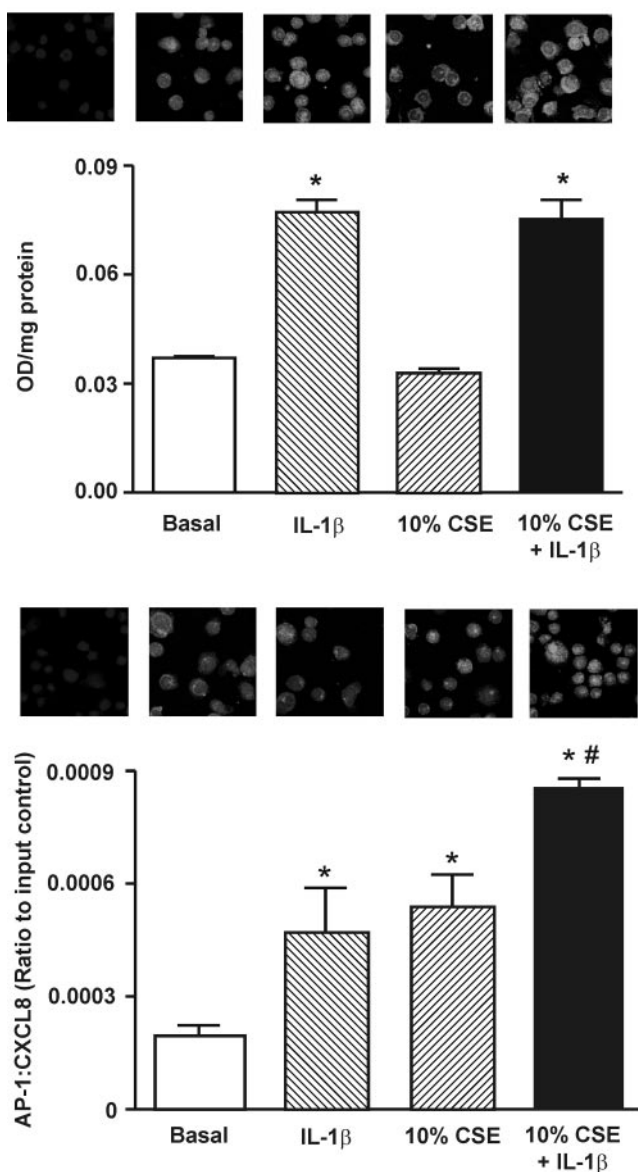


Fig. 9. Expression of AP-1 component c-Jun and NF- κ B component p65 in THP-1 cells after stimulation with CSE or IL-1 β or a combination of both stimuli. The effects of CSE (10%) and IL-1 β (1 ng/ml) were assessed on the nuclear translocation of p65 at 1 h (A), in which the bars represent data obtained from TransAM p65 activation assay. Top, confocal images of p65 nuclear translocation under the same conditions. B, data obtained from a chromatin immunoprecipitation assay for c-Jun binding to the AP-1 motif on the promoter sequence of the human CXCL8 2 h after stimulation. Top, confocal images of phospho-c-Jun nuclear expression under same conditions. Left insets, antibody isotype controls. The data represent the mean \pm S.E.M. for an $n = 9$ from 3 experimental days. *, $p < 0.01$ versus basal and #, $p < 0.01$ versus CSE or IL-1 β alone, as determined by ANOVA followed by Dunnett's post test

mucin was linked to AP-1 activation (Gensch et al., 2004). This is also consistent with the data obtained by Koch and colleagues (2004) that showed an enhanced activation of mitogen-activated kinase over NF- κ B in alveolar macrophages obtained from smokers challenged with LPS. Here, for the first time, we provide definitive evidence that cigarette smoke induces direct physical contact of c-jun with the AP-1 binding site in the promoter region of the CXCL8 gene and that this event, in combination with tonic levels of NF- κ B activation, is crucial for the synergistic release of mature CXCL8 and may account for this protein's biphasic release curve.

In summary, we present novel data that describe and characterize an in vitro cell-based model which mimics the salient characteristics of smoke-induced inflammation in vivo.

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