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Cigarette smoke activates human monocytes by an oxidant - AP-1 signalling pathway: implications for steroid resistance.

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Running title: Cytokines synergise with cigarette smoke in macrophages

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Abbreviations: AP-1, activator protein 1; NFκB, nuclear factor kappa beta; COPD, Chronic obstructive pulmonary disease; PBMC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; SP600125, Anthra[1,9-cd]pyrazol-6(2H)-one1,9-pyrazoloanthrone; MP, N1-Methyl-1,9-pyrazoloanthrone; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; JNK, c-Jun N-Terminal Kinase; GRα, glucocorticoid receptor alpha; CSE, cigarette smoke extract; HO-1, hemeoxygenase; IKK2, NFκB kinase 2.

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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 appears to be a consistent feature. 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 (IL-8). Further, we show for the first time that cigarette smoke synergises with pro-inflammatory cytokines IL-1β and TNFα, 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 the AP-1, but not NFκB, pathway. These observations add significantly to our understanding of smoke as an inflammatory stimulus which has implications for potential the development of treatments of smoking or related disease.

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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 (COPD) 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 in animal models, which is characterised 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 (Mio et al., 1997; Numanami et al., 2003; Russell et al., 2002; Robins at al., 1991). There is no doubt that cigarette smoke is a powerful inflammatory stimulus for the lungs and the immune system. Furthermore, inflammatory cytokines, including IL-1β and TNFα, are elevated in the lungs of smokers, suggesting that smoke may act as a co-activator of cells under these conditions.

Despite a growing body of literature describing the effects of cigarette smoke *in vitro* and *in vivo*, the signalling 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 pathways activated by smoke are likely to be highly relevant to a range of oxidant-dependent inflammatory conditions.

The aims of the current study were three fold. Firstly we have established and characterised a cell based model of cigarette smoke and cytokine induced inflammation. Secondly, we have assessed the effect of therapeutic glucocorticoids on cell activation in our model in order to validate its relevance to human disease. Finally, we have 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 following stimulation of cells with cigarette smoke.

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Methods and Materials

Unless stated otherwise all cell culture reagents were supplied by Invitrogen (Paisley, Renfrewshire, UK).

Cell Culture

THP-1 human monocytes were obtained from the ECACC and cultured in RPMI 1640 containing 10mM GlutaMAX™ and supplemented with 10% FCS, Penicillin/streptamycin 100U/ml, non-essential amino acids. Cells were maintained in a humidified atmosphere at 37°C containing 5% CO_2. THP-1 cells were plated out onto 96 well plates at 500,000/ml in RPMI 1640 (0% FCS content), and left to equilibrate for 1 h before stimulation.

Isolation, Morphological Examination and FACS® Analysis of Blood derived Monocytes and Macrophages.

Isolation of human PBMC. Human PBMC (70% lymphocytes, 30% monocytes), isolated as described previously (Perretti et al., 1999). Briefly, fresh citrated blood was layered on a Fircoll gradient (Histopaque gradient, Histopaque 1077 & Histopaque 1119) and centrifuged (400g; 20°C) for 30 minutes. PBMCs where collected washed with pre-warmed RPMI 1640 and pelleted by centrifugation (450g; 24°C). Cells (10^6/ 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% CO_2) for 2 hours on plastic. Non-adherent 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.

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Monocyte differentiation into macrophages. Isolated monocytes (70%) were differentiated into macrophages using an established protocol (Rossi et al., 1998). Briefly, monocytes (2 x 10^6/ ml) were cultured in 6 well plates and induced to differentiate by incubation with RPMI 1640 containing 10% autologous serum for a period of 7 days (37°C in a 5% CO_2 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 4^0C using PBS containing 1.3mM calcium chloride and 0.2% BSA as buffer. Cells were washed then blocked with normal human IgG (6 mg/ml) and 30 µg/ml of the R-phycoerythrin (RPE)-linked isotype control (IgG2a) monoclonal antibody (clone, DAK-G05) or 30 µg/ml of anti-human CD14 monoclonal antibody (clone, TÜK4), both purchased from DakoCytomation (Glostrup, Denmark) for 1h. Cells were then washed and FACS analysis performed. Cell fluorescence of monocytes gated using forward and side scatter measured in the FL2 channel using a Becton Dickinson FACScan (San Jose, CA, USA). Mean fluorescence intensity (MFI) was calculated by subtracting the MFI 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 (with informed consent). The outside of the tissue was cleaned with NaCl (0.15 M). Tissue was then injected with 10 ml 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

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clear. This resulted in the isolation of $1x10^4$ mononuclear cells/ml of NaCl. Lavage was centrifuged (300g, 10min, 4°C) and pellets pooled in LPHM (Invitrogen, Paisley, UK) containing streptomycin sulphate (100μg/ml), penicillin G (100 IU/ml) and L-glutamine (0.29mg/ml). Macrophages were counted and plated at $5x10^5$ cells per well in 1ml of LPHM. Cells were incubated overnight at 37°C in a humidified atmosphere containing 5.0% CO2, non-adherent cells 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 x 10^5/ml in 96 well plates. To prepare cigarette smoke extract, Four full strength Marlboro cigarettes (filters removed) were combusted through a modified 60ml syringe apparatus and the smoke passed through 100mls of RPMI 1640. Each cigarette yielded 5 draws of the syringe (to 60ml mark), with each individual draw taking approximately 10 seconds to complete. Cigarette smoke extract was then passed through a 0.25μ filter to sterilise and remove particulate matter and was used immediately unless otherwise stated. Cells were treated for between 1 and 24 h with cigarette smoke extract and/or cytokines or LPS (Sigma, Dorset, UK). After this incubation supernatants were collected and stored at -80°C for cytokine determination. In the anti-oxidant experiments (using either N-acetyl cysteine or glutathione; 10-1000 μM; Sigma, Poole, UK) and inhibitor studies (using SP600125 and N1-Methyl-1,9-pyrazoloanthrone (MP); 1-10 μM) all reagents were added 20 minutes before stimulation. In the experiments using glucocorticoids cells were pre-treated 2h prior to stimulation as in human monocytes. This pre-treatment time was chosen using previous data demonstrating that in peripheral blood mononuclear cells and monocytic cell line that 2h is required for receptor activation

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(Paul-Clark et al 2003). Supernatants were collected 24 h post 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 16μM.

Determination of LPS levels in cigarette smoke extract

Smoke extract was made as above. To test LPS contamination a Limulus assay (Eotoxate™, Sigma, Poole, UK) was performed as per the manufacturers instructions on undiluted cigarette smoke extract.

Assessment of cell respiration by MTT.

The effect of IL-1β (1ng/ml), cigarette smoke extract or the co-treatment of cigarette smoke extract and IL-1β on THP-1 cell metabolism was assessed, by measuring the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Poole, UK) to formazan. This was performed following 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, Abingdon, UK). CXCL8 concentrations were measured at 450nm with a reference filter at 550nm and results expressed as pg/ml.

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RT-PCR and Real-Time PCR

Total RNA was isolated from THP-1 monocytes (1- 24 h) post stimulation using the RNeasy Mini Kit (Qiagen, Crawley, UK). cDNA was generated by reverse transcription (RT) 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, Australia) using the SyberGreen PCR Master Mix Reagent Kit (Promega, San Luis Obispo, USA). The sequences of CXCL8 PCR primers were sense 5'-GCCAACACAGAAATTATTGTAAAGCTT and anti-sense 5'-CCTCTGCACCCAGTTTTCCTT'. Primers for GAPDH were sense 5'-ATTCCATGGCACCGTCAAGGCT and antisense 5'- TCAGGTCCA CCACTGACACGT. Primers were used at a concentration of 0.5 μM for real-time. Cycling conditions for real-time PCR (total of 40 cycles used) were as follows: step 1, 15 min at 95 °C; step 2, 15 sec at 94 °C; step3, CXCL8: 25 sec at 60 °C, GAPDH: 25 sec at 64 °C; step 4, 22 sec at 72 °C. Data from the reaction were collected and analysed by the complementary computer software (Corbett Research, Australia). Relative quantitations of gene expression were calculated using standard curves and 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 6-well culture plates and treated with cigarette smoke or IL-1β or IL-1β 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 PBS. For HO-1 cells were lysed using HEPES

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(10mM) containing $MgCl_2$ (3mM) KCL (40mM), Glycerol (5%), Nonidet P-40 (0.3%), PMSF (1mM). For Nrf-2 nuclear and cytosolic proteins were extracted using a recommended nuclear extraction kit (ActiveMotif, Rixensart, Belgium). Protein concentration in both 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, CA, USA) and the signal amplified with a goat anti-rabbit IgG-horseradish peroxidase (1:1000, DakoCytomation, Cambridge, UK). Blots were visualised 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, IL, USA), the blots were probed with β-actin (1:10000; Abcam, Cambridge, UK) for cytosolic HO-1 and Laminin B (1:750; Santa Cruz Biotechnology, CA, USA)for nuclear Nrf-2 which was used to normalise for sample loading.

Transcription factor assay

Active nuclear AP-1 (c-Jun, c-Fos and Fos B) and NFκB (p50, p65, Rel B and p100) levels were measured using a commercial available ELISA based transcription factor assay (ActiveMotif, Rixensart, Belgium) where the relevant response elements was fixed to the base of the well. Target proteins are immobilised 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-1β (1ng/ml) or a combination of the two stimuli for 1 hr. For NF-κB wells were pooled and cells lysed using the lysis buffer supplied by manufactures (ActiveMotif, Rixensart, Belgium). For AP-1 duplicate wells were pooled and subjected to nuclear fraction extraction using the recommended kit (ActiveMotif, Rixensart, Belgium). Protein content was assessed by the Bradford assay. Assays were performed as instructed by the manufacturer using 10µg of total protein. Results are expressed in OD units/µg 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 previously described (Hecht and Grunstein et al., 1999). Cells were resuspended in 200 µl of SDS lysis buffer (50 mM Tris, pH 8.1, 1% SDS, 5 mM EDTA, 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; Active Motif, Brussels, Belgium) or rabbit IgG (DakoCytomation, Glostrup Denmark). 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, 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

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with 70 µg/ml proteinase K (Sigma). DNA was extracted with phenol/chloroform; precipitated with ethanol, 0.3 M NaHCOOH, 20 µg glycogen; and resuspended in 50 µl of Tris-EDTA buffer. Quantitative PCR was performed with 10 µl of DNA sample and 30 cycles. Primer pairs to amplify the AP-1 element within the CXCL8 promoter were as follows: forward (5-AGGAAGTGTGATGACTACGGTT-3) and reverse (5-GAGGAAATTCCACGATTTGC-3). As a control duplicate samples were amplified using primer pairs for Oct-1 element within the CXCL8 promotor, 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 x10^6) were cultured as above in 12 well plates in the presence of CSE, IL-1β or a combination of both or vehicle control (RPMI-1640) for either 0.5 or 2h. Aliquots (10^5 cells) were removed, cytospun (200*g* for 4 min) onto glass slides, air dried, then fixed in ice-cold acetone-methanol (50:50 v/v) at –20^0C for 10 min. Cells were stained for c-jun (rabbit anti-c-jun 1:50; Calbiochem, Nottingham, UK) 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 collected using TCSNT software.

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Statistical Analysis

Statistical analysis was preformed using a One Way analysis of variance (ANOVA) followed by a Dunnet's post test or one-sample T-test for normally distributed data. All data shown is mean ± SEM.

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Results

Quantification and Identification of Human blood monocytes and Macrophages. Monocytes were isolated from whole human blood using a ficoll method and their purity tested using light microscopy and CD14 expression. After cells were allowed to adhere for two hours, PBMC were identified using the monocyte/macrophage marker, CD14, and immunofluorescence quantified by FACS analysis. Monocytes were gated using their forward and side scatter properties. This revealed that 70 ± 8% (n=3; Figure 1A) of the PBMCs stained positively for CD14, with a significant increase of 582 median fluorescent units (MFI; Figure 1D) when compared to the IgG2a isotype control (Figure 1C). These findings were confirmed using light microscopy (inserted panel in Figure 1A) and established that the majority of cells treated were from a monocyte/macrophage lineage. Matched preparations of monocytes from individual donors were further treated with autologous serum for 7 d and the purity of the culture assessed as before. FACS analysis (Figure 1B; CD14) and light microscopy (inserted panel in Figure 1B) revealed that after 7 d, 56 ± 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 Cigarette Smoke Extract (CSE). Initial experiments using smoke revealed that the active component of smoke responsible for the induction of CXCL8 was subject to decay. Importantly cigarette smoke extract (10%) could only optimally induce CXCL8 release from THP-1 cells for up to 1 h post preparation (1167±211 pg/ml 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

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induce CXCL8 from cells continued to decline 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 or not 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 100pg/ml).

CSE Induces the Release of CXCL8 from Human Primary Blood Monocytes, Blood-derived Macrophages and Lung Macrophages: Interactions with IL-1β. We tested the hypothesis that cigarette smoke extract alone and in combination with IL-1β enhances the release of the inflammatory chemokine CXCL8 from primary human monocytes and macrophages. Preliminary experiments revealed that either smoke (1-10%) or IL-1β (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-1β. The combination of cigarette smoke extract (2.5%) and IL-1β (1 ng/ml) caused a synergistic release of CXCL8 in both human PBMCs (Figure 2A) and primary lung macrophages (Figure 2C) when compared to IL-1β 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-1β 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-1β was added simultaneously (Figure 2B).

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

CSE Induces the Release of CXCL8 protein and increased mRNA for CXCL8 in THP-1 Cells: comparisons with IL-1β. 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 pg/ml (versus 52 ± 25 pg/ml at baseline). Concentrations of cigarette smoke extract 20% and above abrogated the increase in CXCL8 levels from THP-1 cells; however, this dramatic decrease was not associated with significant reduction in cellular metabolism as assessed by the MTT assay. Under control culture conditions THP-1 cells released low levels (82 ± 4 pg/ml) of IL-8 over the 24 hour experimental period. However, when cells were treated with increasing concentrations of CSE, IL-8 release was induced in a bell shaped manner (Figure 3). Inhibition of CXCL8 levels at 20% CSE and above couldn't 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 (Figure 4A; $p \leq 0.05$). Similarly when cells were stimulated

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with IL-1β, CXCL8 release was increased at the 8-24 h time points (Figure 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-1β (1 ng/ml) resulted in a synergistic effect on CXCL8 release (Figure 4C). The synergy achieved between smoke and IL-1β was not exclusive to this cytokine since similar results on CXCL8 release (measured at 24h) were obtained with TNFα (Basal, 36±11 pg/ml; TNFα, 61±3 pg/ml; TNFα 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 μM), caused a concentration dependently inhibition of CXCL8 release from THP-1 cells stimulated with smoke, IL-1β or the combination of smoke plus IL-1β.

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

CXCL8 Release Induced by CSE and IL-1β is Functionally Resistant to Inhibition by Clinically Relevant Glucocorticoids. Smoking related inflammation in man is notoriously resistant to inhibition by glucocorticoids. Budesonide and prednisolone are currently used therapeutically in the treatment of responsive inflammatory conditions in man. In order 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.

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Both budesonide and prednisolone caused concentration dependent reductions in the release of CXCL8 induced by cigarette smoke extract (Figure 6A). Interestingly, however neither glucocorticoid had any effect on the CXCL8 release induced by IL-1β (Figure 6B) or by the combination of IL-1β plus cigarette smoke extract (Figure 6C).

Characterisation of the intracellular signalling pathways activated by cigarette smoke extract in the release of CXCL8.

(1) Oxidative Stress. Cigarette smoke consists of more than 10^{16} free radicals per puff (Church and Pryor, 1985). It is therefore predicable that smoke induces oxidative stress within cells. In order 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-1β a clear induction of HO-1 was observed (Figure 7A). With Nrf-2 after 12 h incubation with CSE or IL-1 there was no increase in nuclear expression of this protein. However when CSE (5-20%) was co-incubated with IL-1β a translocation of Nrf-2 from cytosol to the nucleus was observed (Figure 7B).

Next we investigated if the oxidative stress caused by smoke contributed to the induction of CXCL8 release under these conditions. The anti-oxidant N-acetyl cysteine (0.01 -1mM) caused a concentration dependent protection of THP-1 cells from the effects of CSE (Figure 7B). By contrast, NAC had no effect on IL-1β induced CXCL8 release (Figure 7C). Interestingly, the release of CXCL8 induced by cigarette smoke extract plus IL-1β was, like CSE, inhibited by NAC (Figure 7D). In light of the finding that oxidative stress plays an important regulatory role in the

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activation of CXCL8, oxidative sensitive signalling pathways were investigated as possible mechanisms in the effects of cigarette smoke.

(2) c-Jun N-Terminal Kinase (JNK). JNK can be activated by oxidative stress and are therefore likely to be involved in smoke induced activation of cells. Similarly to observations made with NAC and glutathione (data not shown), pharmacological inhibition of JNK using SP600125, inhibited CXCL8 release induced by smoke alone (Figure 8A) and in combination with IL-1β (Figure 8E), but not IL-1β (Figure 8C) at a selective concentration i.e. <10 μM. The control compound N1-Methyl-1,9-pyrazoloanthrone (MP) had no effect on any stimuli at all concentrations tested (1-10 μM; Figures 8 B, D, F). Therefore, these experiments suggest that JNK mediate smoke-induced CXCL8 release from monocytes/macrophages.

(3) NFκB. When cells were stimulated with IL-1β, active nuclear p65 levels were maximally elevated at 1 hour post stimulation (Figure 9A). By contrast NFkB activity was not increased in cells stimulated with cigarette smoke extract (10%; Figure 9A). Co-stimulation with cigarette smoke extract and IL-1β produced similar levels of p65 activation to those seen in cells stimulated with IL-1β alone (Figure 9A). No effect was observed of any treatment on the active levels of either p52 & RelB (data not shown). Inhibition of THP-1 cells with an IKK2 inhibitor, AS602868 (0.3-10 μM), resulted in a marked inhibition of CXCL8 levels by all stimuli used (data not shown).

(4) 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.,

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1991; Smeal et al., 1991). This is consistent with the data obtained in this study where, 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 (not shown) and pronounced c-jun binding to human CXCL8 gene (Figure 9B). Similarly IL-1β increased c-jun nuclear association and direct binding of c-jun to the CXCL8 promoter as determined by chromatin immunoprecipitation assay (Figure 9B). There was a distinct additive effect on c-jun binding to CXCL8 gene when cells were co-stimulated with smoke plus IL-1β (Figure 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 promotor region using antibodies and primers described above.

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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. Firstly we demonstrate that cigarette smoke produces a 'bell-shaped' induction of CXCL8 expression. Secondly, we demonstrate that cigarette smoke can synergise with the inflammatory cytokines TNFα and IL-1β to cause a 'super induction' of CXCL8 release, which importantly, is at the level of new gene transcription. Thirdly, we show that when primed with IL-1β, 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 COPD, is resistant to treatment with steroids (Keating 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 NFκB activation, is involved in the synergistic release of mature CXCL8 in monocytes and macrophages treated with smoke and IL-1β.

Cigarette smoke has been shown to either activate (Rahman et al., 1996) or inhibit the activation (Ouyang et al., 2000; Witherden et al., 1998) of isolated cells in culture. 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 effective concentration of smoke which dictates which overall effect will be seen. Our observations showing smoke stimulates CXCL8 release are particularly robust, having demonstrated this phenomenon in (i) primary moncytes, (ii) monocyte derived macrophages, (iii) lung macrophages and (iv) the pre-monocytic cell line THP-1. We went on to study the mechanisms involved in smoke inducted activation of CXCL8

release, rather than inhibition, because *in vivo* the effect of smoke is pro-inflammatory (Pesci et al., 1998). Interestingly, there is a biphasic response in the induction of CXCL8 gene expression in cells stimulated with smoke or with smoke plus IL-1β with an initial peak at 8h, followed by a decline at 16 h and second peak at 24h. By contrast, when cells were treated with IL-1β alone increased levels of CXCL8 message displayed one peak at 16h. 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 super-induction of CXCL8 by the combination of IL-1β and smoke.

In previous reports the effects of cigarette smoke on inflammatory gene induction have been compared with those of 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 this is a particularly important area of study since in smokers at areas of inflammation, cells are inevitably going to be exposed to both cytokines (including IL-1β) and smoke (Pesci et al., 1998). Importantly we found that when monocytes or lung macrophages were co-stimulated with smoke and IL-1β 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 previously been reported.

Extending these observations, we noted that when cells were co-stimulated with IL-1β plus smoke the resultant release of CXCL8 was completely resistant to inhibition by glucocorticoids. By contrast, when cells were stimulated with smoke alone, the release of CXCL8 was strongly inhibited by glucocorticoids. These observations have directly 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 released disease, cells should be stimulated with the combination of smoke plus IL-1β.

It is not currently clear how cytokine priming confers steroid resistance to a smoke induced inflammatory response. However, given our observations that IL-1β, but not cigarette smoke, induce the activation of NFκB (see below) in our cells, it is tempting to speculate that steroid resistance here is mediated by expression of NFκB inducing the well described phenomenon of NFκB transrepression of GRα (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 NFκB (Bonizzi and Karin, 2004) and AP-1 pathways (Saulian and Karin, 2002). The current literature describing the effects of smoke on AP-1 and NFκB 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 NFκB (Moodie et al., 2004). Similarly smoke may activate (Gensch et al., 2004), inhibit the activation (Lann et al., 2004) or no affect AP-1 mediated signalling (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, where activation or inhibition of target genes has been equally described. In the current study we initially identified the involvement of oxidant stress in smoke induced CXCL8 release. Oxidant stress is increasingly recognised as a trigger for the activation of either NFκB or AP-1 (Maziere et al., 1999). However, our data shows that, in our cells, smoke does not cause a substantial activation of NFκB. The effects

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here are in contrast to those of IL-1β, which predictably increased NFκB activity in our cells. However, the IKK2 inhibitor AS602868 substantially reduced CXCL8 levels after activation with all stimuli. These observations effectively rule out NFκB in the super-induction of CXCL8 in monocytes and macrophages by smoke and IL-1β in our system. However, there is some requirement for tonic levels of NFκB in the activation and release of CXCL8 from human macrophages.

Reactive oxidative species has been demonstrated to upregulate 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 where the release of mucin was linked to AP-1 activation (Gench et al., 2004). This is also consistent with the data obtained by Koch and colleagues that showed and enhanced activation of mitogen-activated kinase over NFκB in alveolar macrophages obtained from smokers challenged with LPS (Koch et al., 2004). 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 NFκB activation, is crucial for the synergistic release of mature CXCL8 and may account for this proteins biphasic release curve.

In summary we present novel data which describes and characterises an *in vitro* cell based model which mimics the salient characteristics of smoke induced inflammation *in vivo*.

References

Ambrose JA and Barua RS (2004) The pathophysiology of cigarette smoking and cardiovascular disease: an update. *J Am Coll Cardiol* **43**:1731-1737.

Bishop-Bailey D, Larkin SW, Warner TD, Chen G and Mitchell JA (1997) Characterization of the induction of nitric oxide synthase and cyclo-oxygenase in rat aorta in organ culture. *Br J Pharmacol* **121**:125-133.

Bonizzi G and Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* **25**:280-288.

Burns DM (2003) Tobacco-related diseases. *Semin Onco. Nurs* **19**: 244-9.

Church DF and Pryor WA (1985) Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* **64**:111-126.

Culpitt SV, Rogers DF, Shah P, De Matos C, Russell RE, Donnelly LE and Barnes PJ (2003) Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **167**:24-31.

Favatier F and Polla BS (2001) Tobacco-smoke-inducible human haem oxygenase-1 gene expression: role of distinct transcription factors and reactive oxygen intermediates. *Biochem J* **353**:475-482.

Gensch E, Gallup M, Sucher A, Li D, Gebremichael A, Lemjabbar H, Mengistab A, Dasari V, Hotchkiss J, Harkema J and Basbaum C (2004) Tobacco smoke control of mucin production in lung cells requires oxygen radicals AP-1 and JNK. *J Biol Chem* **279**:39085-39093.

Hecht A and Grunstein M (1999) Mapping DNA interaction sites of chromosomal proteins using immunoprecipitation and polymerase chain reaction. *Methods Enzymol* **304**:399-414.

Ito K, Barnes PJ and Adcock IM (2000) Glucocorticoid receptor recruitment of histone deacetylase-2 inhibits interleukin-1ß-induced histone H4 acetylation on lysine 8 and 12. *Mol Cell Biol* **20**:6891-6903.

Keatings VM, Jatakanon A, Worsdell YM and Barnes PJ (1997) Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med* **155**:542-548.

Koch A, Giembycz M, Stirling RG, Lim S, Adcock I, Wabermann K, Erdmann and Chung KF (2004) Effect of smoking on MAP kinase-induced modulation of IL-8 in human alveolar macrophages. *Eur Respir J* **23**: 805-812.

Laan M, Bozinovski S and Anderson GP (2004) Cigarette smoke inhibits lipopolysaccharide-induced production of inflammatory cytokines by suppressing the activation of activator protein-1 in bronchial epithelial cells. *J Immunol* **173**:4164-4170.

Lee K and Esselman WJ (2002) Inhibition of PTPs by H(2)O(2) regulates the activation of distinct MAPK pathways. *Free Radic Biol Med* **33**:1121-1132.

McKay LI and Cidlowski JA (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signalling pathways. *Endocr Rev* **20**:435-459.

Maziere C, Conte MA, Degonville J, Ali D and Maziere JC (1999) Cellular enrichment with polyunsaturated fatty acids induces an oxidative stress and activates the transcription factors AP1 and NFkappaB. *Biochem Biophys Res Commun* **265**:116-122.

Mio T, Romberger D, Thompson A, Robbins R, Heires A and Rennard S (1997) Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med* **155**:1770-1776.

Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bauter MR, Kilty I and Rahman I (2004) Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. *Faseb J* **18**:1897-1899.

Murray CJL, Lopez AD, Metthers CD and Stein C (2001) The Global burden of disease 2000 project: global programme on evidence for healthy policy discussion paper number **36**. *Geneva: WHO.*

Numanami H, Koyama S, Nelson DK, Hoyt JC, Freels JL, Habib MP, Amano J, Haniuda M, Sato E and Robbins RA (2003) Serine Protease Inhibitors Modulate Smoke-Induced Chemokine Release From Human Lung Fibroblasts. *Am J Respir Cell Mol Biol* **29:**613-619**.**

Ouyang Y, Virasch N, Hao P, Aubrey MT, Mukerjee N, Bierer BE and Freed BM (2000) Suppression of human IL-1beta, IL-2, IFN-gamma, and TNF-alpha production by cigarette smoke extracts. *J Allergy Clin Immunol* **106**:280-287.

Paul-Clark MJ, Roviezzo F, Flower RJ, Cirino G, Del Soldato P, Adcock IM, and Perretti M (2003) Glucocorticoid receptor nitration leads to enhanced anti-inflammatory effects of novel steroid ligands. *J Immunol* **171**:3245-3252.

Perretti M, Wheller SK, Flower RJ, Wahid S and Pitzalis C (1999) Modulation of cellular annexin I in human leukocytes infiltrating DTH skin reactions. *J Leukoc Biol* **65**:583-589.

Pesci A, Balbi B, Majori M, Cacciani G, Bertacco S, Alciato P and Donner CF (1998) Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J* **12**:380-386.

Pi J, Qu W, Reece JM, Kumagai Y and Waalkes MP (2003) Transcription factor Nrf2 activation by inorganic arsenic in cultured keritinocytes: involvement of hydrogen peroxide. *Exp Cell Res* **290**: 234-245.

Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E and Woodgett JR (1991) Phosphorylation of c-jun mediated by MAP kinases. *Nature* **353**:670-674.

Rahman I, Smith CA, Lawson MF, Harrison DJ and MacNee W (1996) Induction of gamma-glutamylcysteine synthetase by cigarette smoke is associated with AP-1 in human alveolar epithelial cells. *FEBS Lett* **396**:21-25.

Rahman I, Bel A, Mulier B, Donaldson K and MacNee W (1998) Differential regulation of glutathione by oxidants and dexamethasone in alveolar epithelial cells. *Am J Physiol* **275**:L80-L86.

Robbins RA, Nelson KJ, Gossman GL, Koyama S and Rennard SI (1991) Complement activation by cigarette smoke. *Am. J. Physiol.* **260**:L254-L259.

Rossi AG, McCutcheon JC, Roy N, Chilvers ER, Haslett C and Dransfield I (1998) Regulation of Macrophage Phagocytosis of Apoptotic Cells by cAMP. *J Immunol* **160**:3562-3568.

Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J and Barnes PJ (2002) Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* ***26***:602-609.

Shaulian E, and Karin M (2002) AP-1 as a regulator of cell life and death. *Nat Cell Biol* **4**:E131-E136.

Shishodia S, Potdar P, Gairola CG, and Aggarwal BB (2003) Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF-kappaB activation through inhibition of IkappaBalpha kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1. *Carcinogenesis* **24**:1269-1279.

Smeal T, Binetruy B, Mercola DA, Birrer M and Karin M (1991) Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature* **354**:494-496.

Stanford SJ, Walters MJ, Hislop AA, Haworth SG, Evans TW, Mann BE, Motterlini R and Mitchell JA (2003) Heme oxygenase is expressed in human pulmonary artery smooth muscle where carbon monoxide has an anti-proliferative role. *Eur J Pharmacol* **473**:135.

van der Vaart H, Postma DS, Timens W and ten Hacken NH (2004) Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* **59**:713-721.

Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U and. Tetley TD (2004) Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase. *A. J Respir Cell Mol Biol* **30:**500-509.

Footnotes

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Figure legends

Figure 1. Characterisation of cell types by FACS analysis and CD14 expression in adherent PBMC's and blood derived macrophages. PBMC's 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 hand-side quadrant of panels (A) and (B). Panel (A) shows FACS analysis of PBMCs that were allowed to adhere for 2 h with the inserted panel showing morphology by light microscopy of the same cells, whereas Panel (B) shows PBMCs that have been incubated with autologus serum for 7 days with the inserted panel showing morphology by light microscopy of the same cells. Panel (C) show cells that have been treated with IgG2a isoype (clone, DAK-G05) control linked to R-Phycoerythrin (RPE) and Panel (D) shows cells, that have adhered to plastic for 2 h, and treated with a monoclonal anti-human CD-14 antibody (clone, TÜK4) linked to RPE. Diagrams are representative of 3 different subjects carried out over 3 different experimental days.

Figure 2. Characterisation 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-1β (1ng/ml) or a combination of both were assessed on CXCL8 release by (A) human peripheral blood monocytes or (B) human autologous serum derived blood macrophages or (C) human lung tissue derived macrophages. The data is the mean ± SEM for cells from n=3 separate donors. Synergy is shown by #.

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Figure 3. The effect of increasing concentrations of CSE on IL-8 release from THP-1 cells. Cells were stimulated with CSE concentrations from 1-30% for 24 hrs. Cell free supernatant was assessed for IL-8 by ELISA. CSE induced IL-8 release from THP-1 monocytes in a bell shaped manner. At CSE concentrations below 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 above IL-8 release was inhibited. N= 9 from 3 experimental days.

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

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

Figure 6. Glucocorticoids were unable to inhibit CXCL8 release from THP-1 cells after stimulation with a combination of CSE or IL-1β. The effects of budesonide or prednisolone (both 0.3-10 μM) were assessed on CXCL8 release from THP-1 cells stimulated with either (A) 10% CSE or (B) IL-1β (1ng/ml) or (C) a combination of 10% CSE and IL-1β (1ng/ml). The data represents the mean ± SEM for an n=9 from 3

experimental days. *, $p<0.01$ as determined by ANOVA followed by Dunnets post test.

Figure 7. Assessment of oxidative stress in THP-1 cells induced by CSE and IL-1β. The effect of LPS (10 µg/ml), IL-1β (1ng/ml), CSE (5-20%) and a combination of CSE (5-20%) and IL-1β (1ng/ml) was assessed on (A) expression of the oxidative sensitive protein HO-1 with β Actin protein loading control and (B) nuclear expression of the redox-sensitive transcription factor Nrf-2 with laminin B as nuclear protein loading control. Since the oxidative stimulus CSE and in combination with IL-1β induced HO-1 expression the effects of the anti-oxidant N-acetyl cysteine (NAC) was assessed on CXCL8 release from THP-1 cells treated with either (C) 10% CSE alone or (D) IL-1β (1ng/ml) or (E) a combination of 10% CSE and IL-1β (1ng/ml). Please note the differences in scales between parts (C), (D) and (E). The data represents the mean ± SEM for an n=9 from 3 experimental days. *, $p<0.01$ as determined by ANOVA followed by Dunnets post test.

Figure 8. Concentration dependent inhibition of CXCL8 by the JNK kinase inhibitor SP600125. The effects of SP600125 (1-10 µM) and control compound N1-Methyl-1,9-pyrazoloanthrone (MP; 1-10 µM) added 20 min pre-activation were assessed on CXCL8 release after 24 h from THP-1 cells stimulated with either (A and B) 10% CSE or (C and D) IL-1β (1ng/ml) or (E and F) a combination of 10% CSE and IL-1β (1ng/ml). The data represents the mean ± SEM for an n=9 from 3 experimental days. *, $p<0.01$ as determined by ANOVA followed by Dunnets post test.

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Figure 9. Expression of AP-1 component c-Jun and NF-kB component p65 in THP-1 cells after stimulation with CSE or IL-1β or a combination of both stimuli. The effects of CSE (10%) and IL-1β (1ng/ml) were assessed on the nuclear translocation of p65 at 1h (A), where the bars represent data obtained from TransAM® p65 activation assay and inserted panels above represent confocal images of p65 nuclear translocation under same conditions. (B) represents data obtained from a ChIP assay for c-jun binding to the AP-1 motif on the promotor sequence of the Human CXCL8, 2h after stimulation. Inserted panels above represent confocal images of phospho-c-jun nuclear expression under same conditions. The left hand inserted panel for both parts of the Figure represent antibody isotype controls. The data represents the mean ± SEM for an n=9 from 3 experimental days. *, $p<0.01$ vs basal and #, vs CSE or IL-1β alone as determined by ANOVA followed by Dunnets post test

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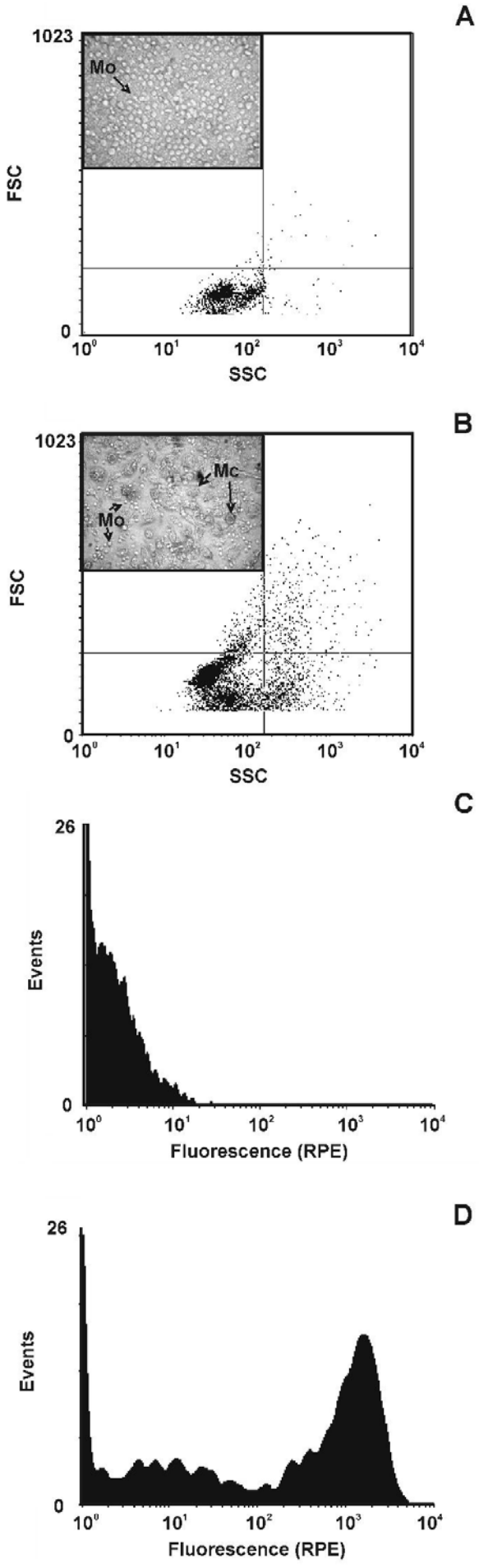

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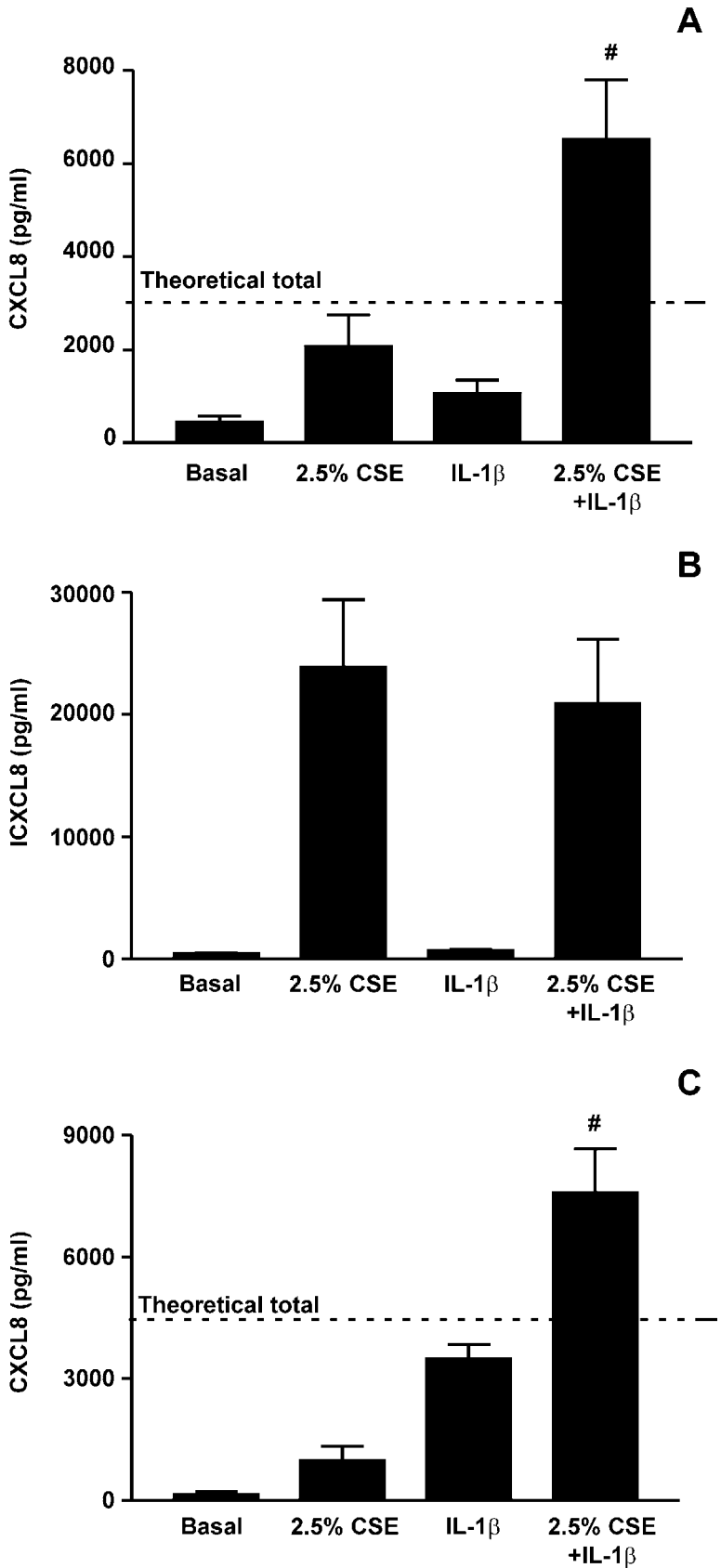

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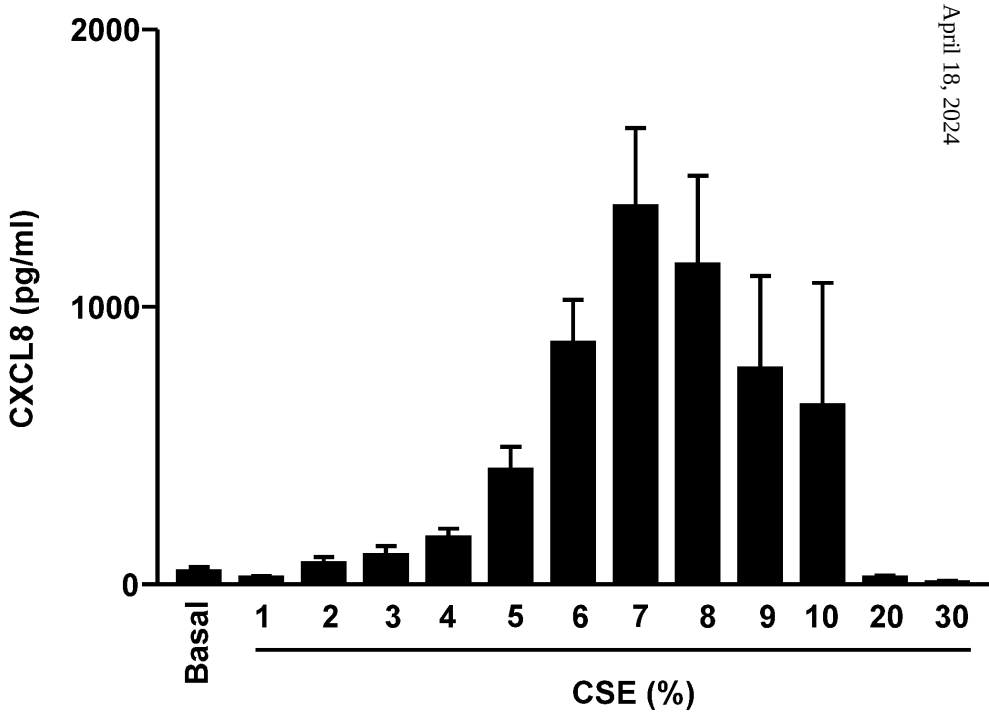
Mol 012591 - Figure 3



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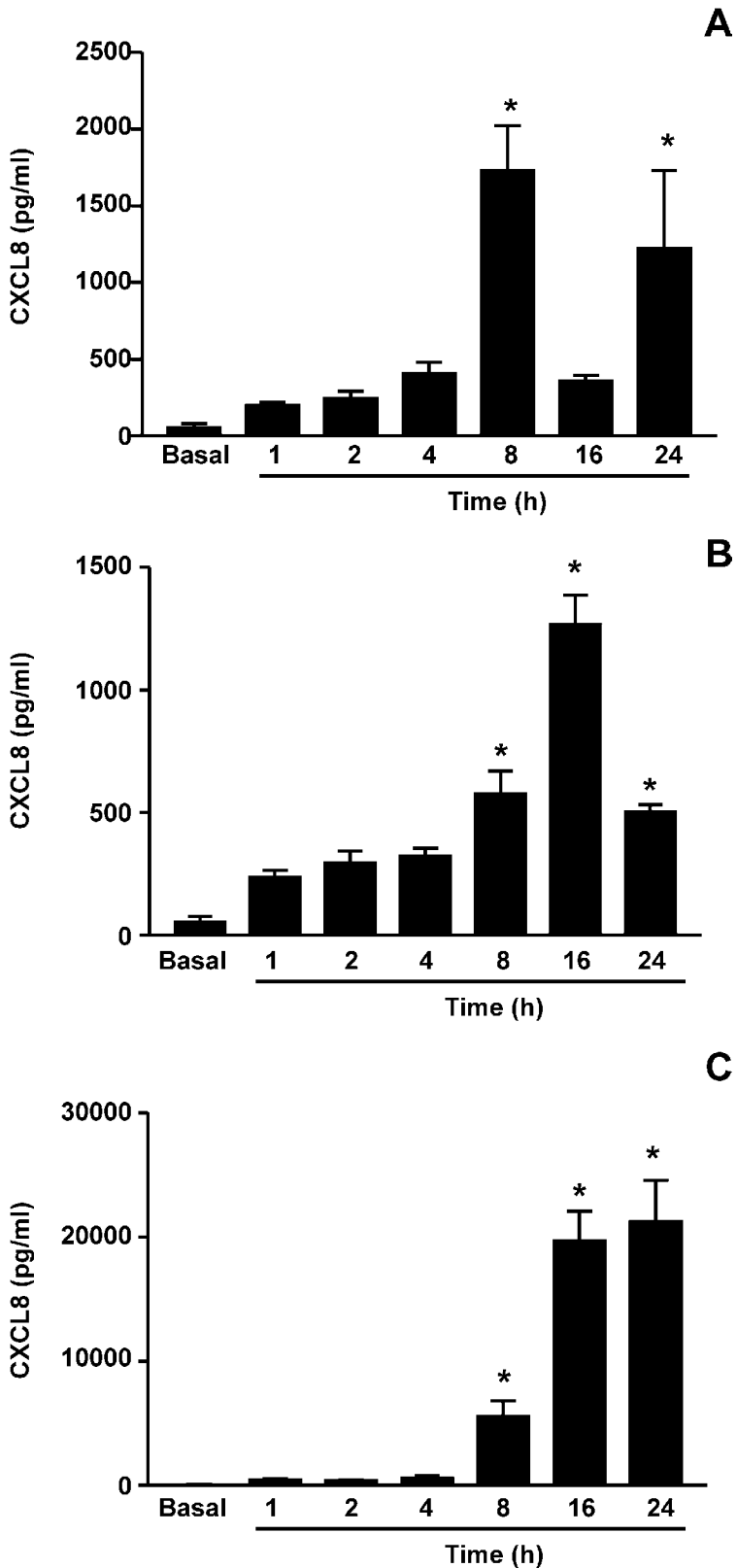

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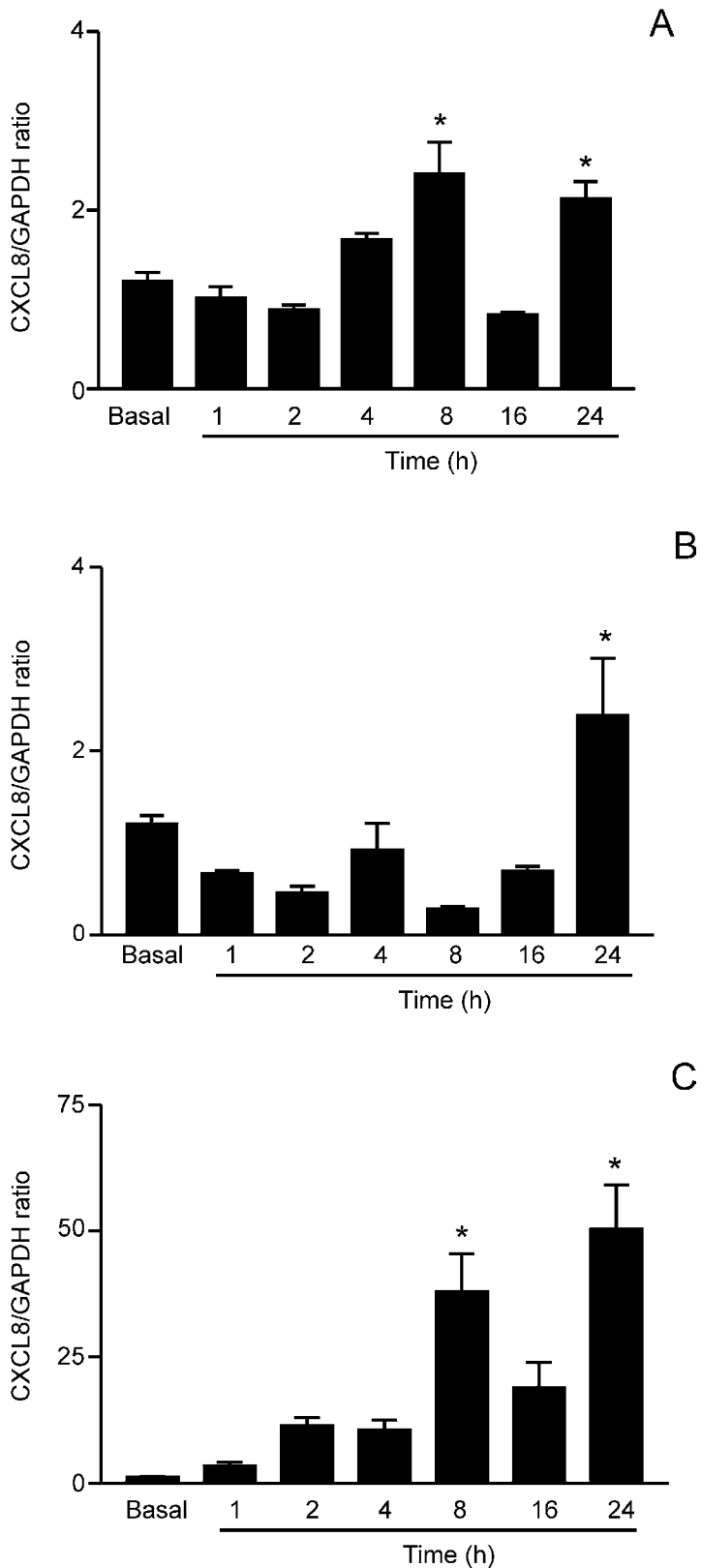

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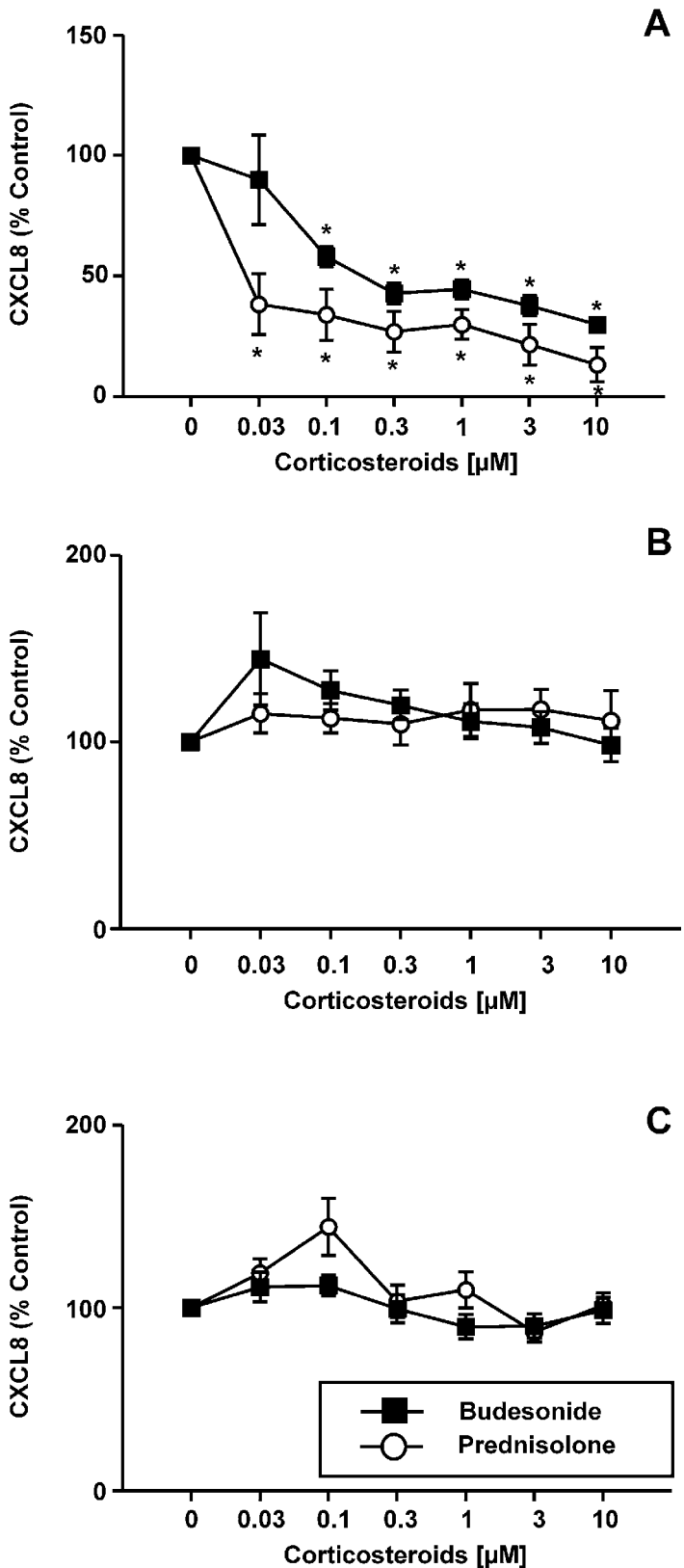

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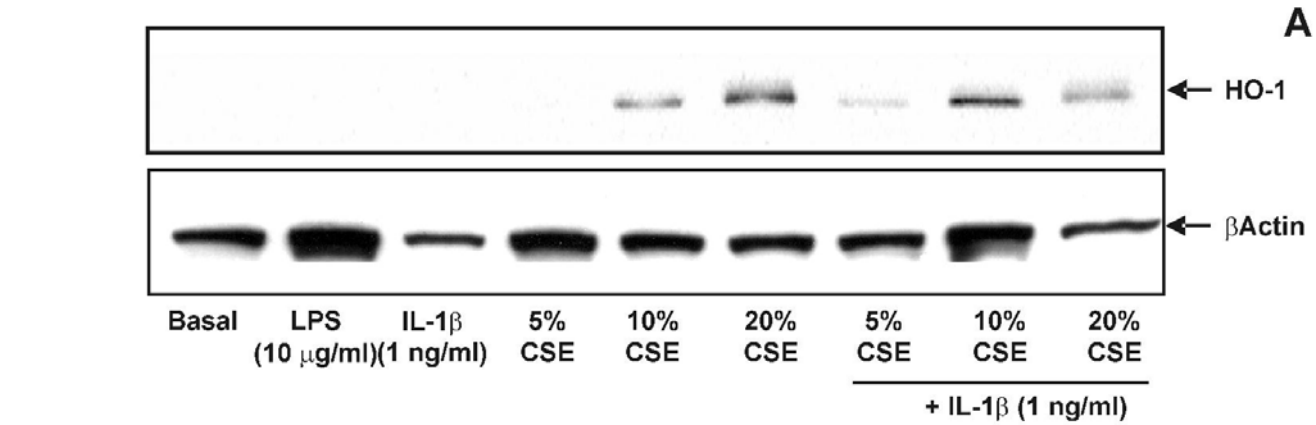
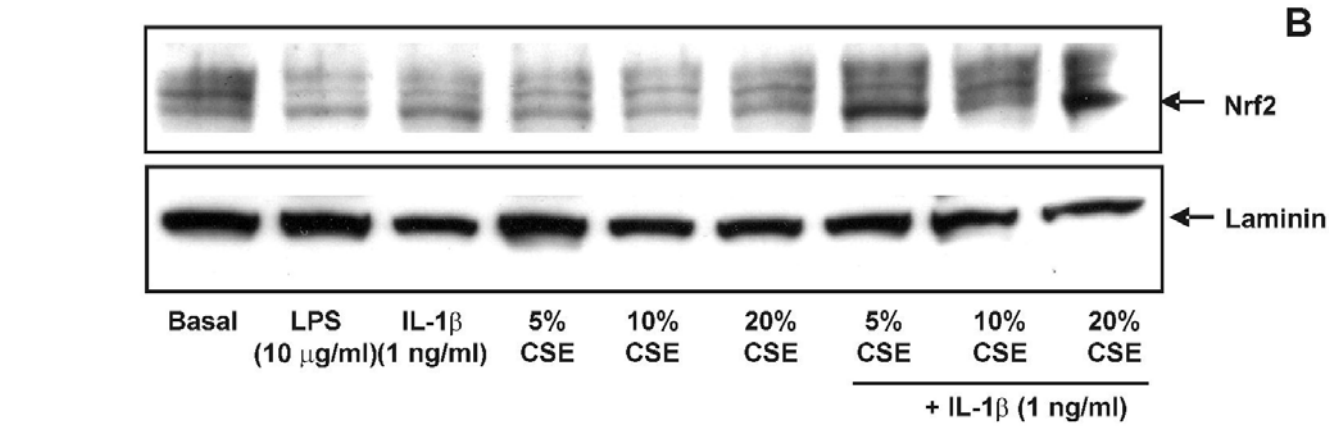
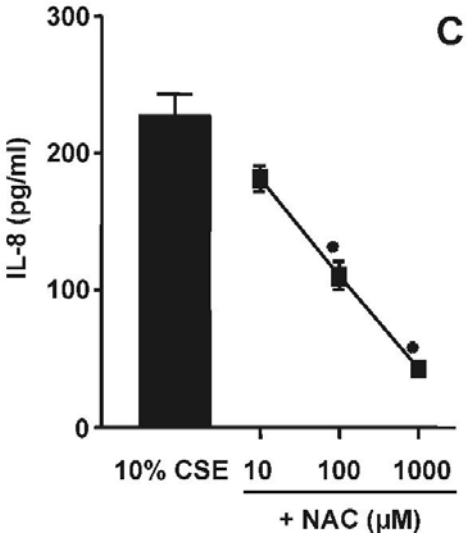
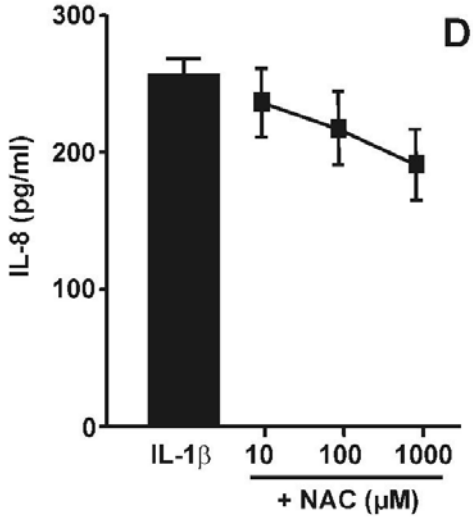
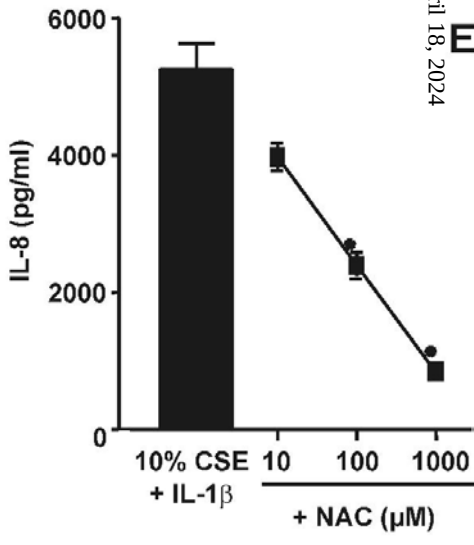
Mol 012591 - Figure 7











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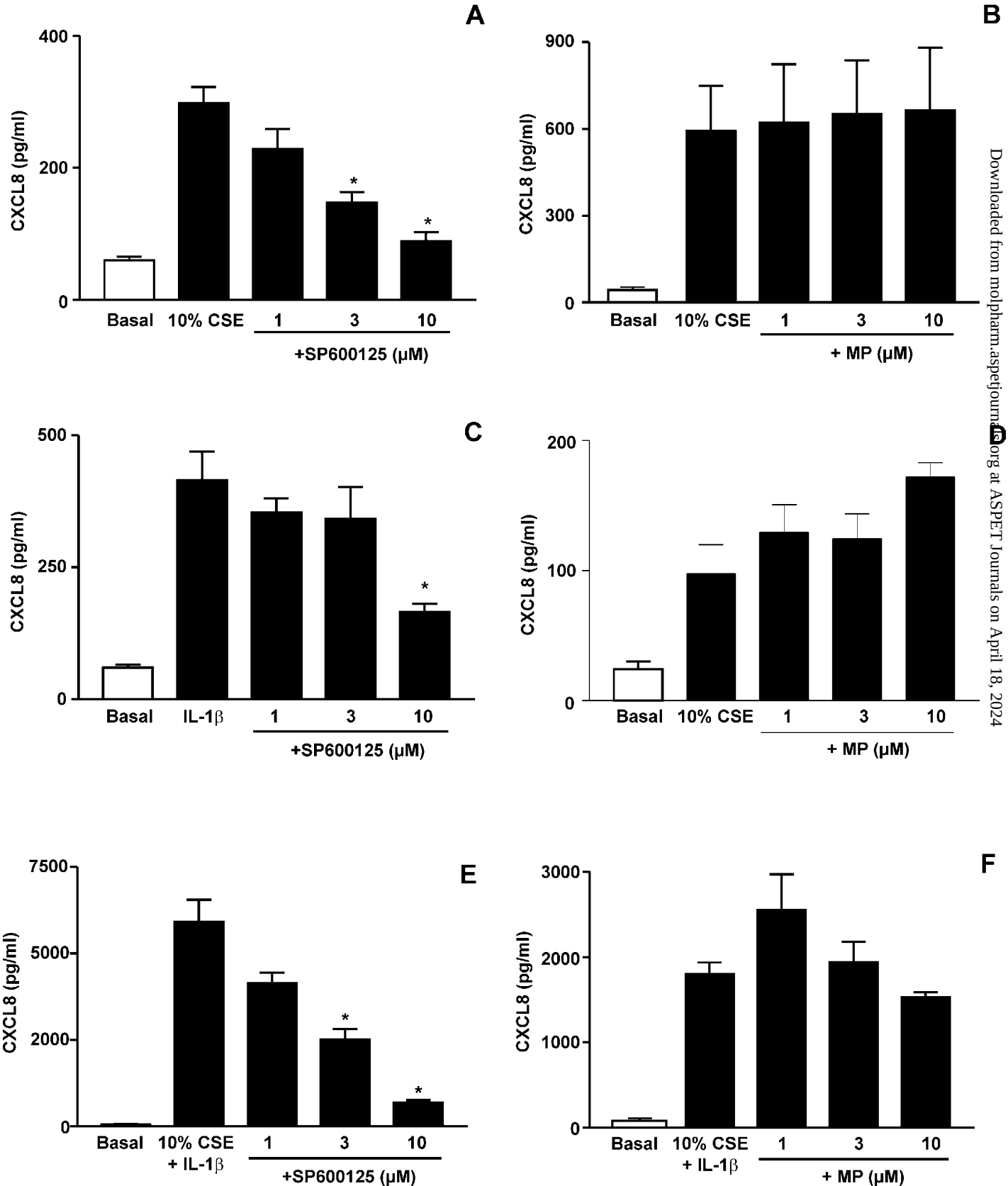

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