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Potential role of c-Jun NH_2-terminal kinase in allergic airway inflammation and remodelling: effects of SP600125

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Abstract

Asthma is a chronic inflammatory disease of the airways associated with structural changes such as increased airway smooth muscle mass, which may contribute to impairment of lung function. To determine whether c-Jun NH_2-terminal kinase (JNK) of the mitogen-activated protein kinase signalling pathway participated in these changes, the effects of an inhibitor, SP600125 (anthra [1, 9-cd] pyrazole-6 (2H)-one), were examined in a murine model of chronic airway inflammation and remodelling. Mice sensitised to ovalbumin were exposed to ovalbumin aerosol and were treated with SP600125 [30 mg kg^{-1} intraperitoneal (i.p.)] on days of exposure. SP600125 significantly reduced eosinophil and lymphocyte numbers in bronchoalveolar lavage fluid, suppressed eosinophilic inflammation within the bronchial submucosa, inhibited goblet cell hyperplasia, and increased airway smooth muscle cell number in allergen-exposed mice. SP600125 also inhibited allergen-induced increase in bronchial responsiveness. SP600125 inhibited JNK activity in the challenged lungs. Although SP 600125 may also have other effects, we conclude that c-Jun NH_2-terminal kinase may play a role in allergen-induced inflammation and remodelling associated with bronchial hyperresponsiveness.

Keywords: Asthma; c-Jun NH_2-terminal kinase; Airway inflammation; Airway smooth muscle; Bronchial responsiveness

1. Introduction

Asthma is a chronic inflammatory disease of the airways characterised by the presence of a cellular inflammatory process consisting of eosinophils, lymphocytes, macrophages, neutrophils, and mast cells within the bronchial mucosa and by bronchial hyperresponsiveness (Bradley et al., 1991). In addition, there are alterations to the structural cells of the airways consisting of increased airway smooth muscle mass, goblet cell hyperplasia, and thickening of the basement membrane (Jeffery, 2001). The increased airway smooth muscle mass, consisting of both airway smooth muscle cell hyperplasia and hypertrophy (Ebina et al., 1993), contributes to increased thickening of the airway wall, which may be an important determinant of excessive airway lumen narrowing (James et al., 1989). The mechanisms underlying these changes in airway smooth muscle are complex and may involve proinflammatory and mitogenic cytokines and growth factors.

Mitogen-activated protein kinases are a family of kinases that phosphorylate specific sites of target protein substrates to regulate different cellular activities. In mammals, three major mitogen-activated protein kinase subfamilies that differ in substrate specificity and response have been identified; c-Jun NH_2-terminal kinase, extracellular regulating kinase, and p38 kinase (Johnson and Lapadat, 2002). Ten c-Jun NH_2-terminal kinase isoforms have been identified, which are encoded by three genes, c-Jun NH_2-terminal kinase (JNK)-1, JNK-2, and JNK-3 (Gupta et al., 1996).

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JNK-1 and JNK-2 have been identified in the lungs of mammals, while JNK-3 has been located to the brain (Bennett et al., 2001). A number of stimuli such as certain inflammatory cytokines, osmotic shock, and ionising radiation (Whitmarsh and Davis, 1996; Mitsutake et al., 2001) can activate c-Jun NH_2-terminal kinase leading to the dual phosphorylation of specific sites (serine 63 and serine 73) on the amino-transactivation domain of c-*jun*, a component of the transcription factor, activator protein-1. Many cytokines involved in allergic inflammation and the remodelling processes are regulated by transcription factors, such as nuclear factor-κB, and activator protein-1 (Barnes and Adcock, 1998). Activator protein-1 binding sites have been identified in promoter regions of proinflammatory genes, including T helper type 2 cytokines (Rooney et al., 1995; Mori et al., 2000) adhesion molecules (Korenaga et al., 1997) and growth factors (Hata et al., 2000). In addition, airway mucus production may also be under the control of activator protein-1 since the gene of an important component of mucus, MUC5B, contains a putative binding site for this transcription factor (Van et al., 2000). Recently, the inhibition of activator protein-1 by a small molecule inhibitor in ovalbumin-sensitised and exposed mice led to attenuation of airway eosinophilia and mucus production and abrogation of allergen-induced bronchial hyperresponsiveness (Henderson et al., 2002).

SP600125 is a reversible ATP-competitive inhibitor, exhibiting more than 20-fold selectivity for JNK-1, -2, and -3 against related mitogen-activated protein kinases such as extracellular regulating kinase and p38 kinase (Bennett et al., 2001). Using a model of chronic allergen exposure in sensitised Balb/c mice, we have examined the involvement of c-Jun NH_2-terminal kinase in airway eosinophilic inflammation, airway smooth muscle and goblet cell hyperplasia, and bronchial hyperresponsiveness.

2. Methods

2.1. Animals, sensitisation procedures, and allergen exposure

Pathogen-free male BALB/c mice (6–8 weeks; 22–26 g) were purchased from Harlan-Orlac (Bicester, UK). Animals were housed in a caging system (Maximiser, Theseus caging system, Hazelton, PA, USA), and U.K. Home Office guidelines for animal welfare based on Animals (Scientific Procedures) Act 1986 were strictly observed. Animals were sensitised on Day 0 by intraperitoneal (i.p.) injection containing 10 μg ovalbumin (Chicken Egg Ovalbumin, Grade V) and 2 mg $Al(OH)_3$ (BDH, Dorset, UK) in 0.2 ml sterile phosphate-buffered saline (PBS). On Day 14, the mice were given an i.p. booster injection of the same allergen/$Al(OH)_3$ mix.

Seven days after the second sensitisation, mice were challenged with aerosolised ovalbumin (50 mg/ml in PBS) or PBS alone. Ovalbumin aerosol exposure was performed by placing animals in a 6.5-l Plexiglass chamber connected to an ultrasonic nebulizer (DeVilbiss, UK). The aerosol was circulated through the chamber using a ventilator (Harvard Apparatus) generating an aerosol mist. Animals were exposed to allergen for 20 min per day for five consecutive days for three weeks, totalling 15 challenges. Seventy-two hours after the last aerosol exposure, bronchial responsiveness to acetylcholine was measured, and bronchoalveolar lavage fluid and lung tissue were obtained.

2.2. Protocol

Four groups of sensitised mice to ovalbumin and $Al(OH)_3$ were studied:

(1) Vehicle-treated PBS-exposed animals (n=8): mice were treated with vehicle 2 h prior and 8 h following PBS exposure.
(2) SP600125-treated PBS-exposed animals (n=8): mice were treated with SP600125 2 h prior and 8 h following PBS exposure.
(3) Vehicle-treated ovalbumin-exposed animals (n=8): mice were treated with vehicle 2 h prior and 8 h following ovalbumin exposure.
(4) SP600125-treated and ovalbumin-exposed (n=9): mice were treated with SP600125 2 h prior and 8 h following ovalbumin exposure.

2.2.1. SP600125

SP600125 (anthrax [1,9-cd] pyrazole-6 (2H)-one) is a novel c-Jun NH_2-terminal kinase inhibitor synthesized by Department of Chemistry at Signal Research Division of Celgene, San Diego, CA. SP600125 was prepared as a 30 mg kg^{-1} solution in a vehicle of 10% ethanol, 15% Cremophor-El, 30% polyethylene glycol-400, and 20% propylene glycol in sterile saline. Administration of SP600125 at 30 mg kg^{1} intravenously (i.v.) has been demonstrated to be efficacious in a mouse model of endotoxin-induced inflammation, with the inhibition of lipopolysaccharide-induced tumor necrosis factor-α serum levels (Bennett et al., 2001). Therefore, mice were injected i.p. with either vehicle or SP600125 (30 mg kg^{-1}; 0.16 ml volume) 2 h prior to and 8 h following each allergen or PBS exposure.

2.3. Measurement of bronchial responsiveness

Mice were anaesthetised with an i.p. injection of anaesthetic solution containing midazolam (Roche Products, Welwyn Garden City, UK) and Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone; Janssen Animal Health, Wantage, UK) in distilled water. Animals were tracheostomised with plastic tubing and placed on a ventilator (Mini Vent type 845, Hugo Sach Electronic, Germany) via a tracheostomy tube at a rate of 250 breaths/

min and a tidal volume of 250 μl. Mice were monitored in a whole body plethysmograph with a pneumotachograph connected to a transducer (Infodisp, Herts, UK). Transpulmonary pressure was assessed via an oesophageal catheter connected to a transducer (Infodisp). The signals from the transducers were digitised with an analogue digital board connected to a Microsoft computer and analysed with Infodisp software, which is programmed to instantaneously calculate pulmonary resistance and compliance. After recording stable baseline lung resistance, increasing concentrations of acetylcholine chloride (Sigma, Dorset, UK; 4–256 mg/ml) were administered by aerosol for 45 s at each concentration with an ultrasonic nebulizer, and lung resistance was recorded for a 5-min period. Each concentration of acetylcholine was separated by 10-min intervals with hyperinflation of the lung to twice the tidal volume, performed by blocking the outflow of the ventilator manually (to ensure a constant volume history prior to each concentration). The maximum lung resistance values were expressed as percentage change from baseline lung resistance measured after PBS aerosol. The provocative concentration (PC_{200}) of acetylcholine needed to increase lung resistance 200% above PBS baseline was also calculated by interpolation of the log concentration–lung resistance curve from individual animals, and $-\log PC_{200}$ was taken as a measure of bronchial responsiveness.

2.4. Bronchoalveolar lavage

Immediately after the assessment of bronchial responsiveness, mice were killed using an overdose of pentobarbitone (60 mg/kg i.p.). The trachea was catheterised. Six separate 0.5-ml aliquots of PBS were injected into the tracheal cannula and collected as the bronchoalveolar lavage fluid. The lavage fluid was centrifuged at 1500 rpm for 15 min at 4 °C. The supernatant was aliquoted and stored at −20 °C. The remaining cell pellet was resuspended in 0.5 ml Hanks balanced solution. Total cell counts were performed by haemocytometry by adding 10 μl of the cell suspension to 90 μl of Kimura stain. Differential cell counts were prepared on cytospins (Shandon, UK) by adding 50–200 μl of the cell suspension and centrifuging at 500 rpm for 5 min. Cytospins were stained with May-Grűnewald-Giemsa stain. Differential cell counts on at least 200 cells were done for enumeration of eosinophils, macrophages, T lymphocytes, and neutrophils in each sample.

2.5. Collection of lung tissue

The heart and lungs were perfused in situ with 0.8 ml PBS/Optimum Cutting Temperature Compound (1:1). The inflated heart and lungs were removed from the thoracic cavity, and tissue blocks were mounted in Optimum Cutting Temperature embedding medium on cork disks and snap-frozen in *iso*-pentane cooled by liquid nitrogen. Frozen-tissue blocks were kept in the −20 °C freezer before use. All lung tissue sections were cut (6μm) in a cryostat (Bright OTF Cryostat, Bright Instruments, Huntingdon) alongside the main intrapulmonary bronchus and mounted on glass slides precoated with poly-L-lysine for improved binding. Sections were stored at −20 °C before being used.

2.5.1. Haematoxylin and eosin staining of airway smooth muscle cells

Sections were fixed in 10% formalin-buffered saline for 10 min and washed in distilled water for 2 min and then transferred to 20% haematoxylin (BDH) for 30 s and immediately washed in tap water for 2–3 min. Sections were then transferred to 1% eosin (BDH) for 10 s and immediately washed in tap water for 2–3 min (changing water every minute). Finally, sections were dehydrated through graded alcohols (70%, 90%, and 100%) cleared through xylene (BDH) and mounted with DePex mounting solution (Raymond Lamb, UK). Sections were coded and read; counts were expressed as number of cells per millimetre of basement membrane.

2.5.2. Quantification of airway smooth muscle cells

Quantification of airway smooth muscle cells were performed using a Zeiss Light microscope (Welwyn Garden City). Using an image analysis system (KS 300 model, Imaging Associates, Thame, UK), the whole airway was visualised with an ×5 objective lens. The internal perimeter was measured as the basement membrane length. The airway was then visualised under a ×40 objective lens, and the number of elongated airway smooth muscle cell nuclei stained with haematoxylin was counted within 50 μm of the basement membrane. Counts were expressed as the number of airway smooth muscle cells per millimetre of basement membrane.

2.5.3. Periodic Acid Schiff and Alcian Blue staining for goblet cells

Sections were fixed in acetone for 10 min followed by paraformaldehyde for a further 10 min. Sections were then washed in distilled water for 2 min before being immersed in a 4% Alcian Blue solution (pH 2.5). After a 5-min period, the sections were washed in running tap water. Sections were then treated with 1% aqueous periodic acid for 5 min. Following a rinse in distilled water, sections were incubated with Schiff's reagent (Sigma) for 4 min and washed under running tap water. Sections were finally dehydrated through graded alcohols (70%, 90%, and 100%) before being cleared through xylene and mounted. Sections were counted under a light microscope. The numbers of blue/purple goblet cells were counted around the internal perimeter of the airway. Counts are expressed as goblet cells per millimetre of epithelial membrane.

2.5.4. Cyanide-resistant eosinophil peroxidase staining

For the detection of eosinophils in mouse lung tissue, a cyanide-resistant eosinophil peroxidase stain was used

(Boyce et al., 1995). Sections were allowed to dry in air and fixed in 4% picric acid at room temperature (10 s) followed by rinsing in tap water to remove excess fixative. Sections were transferred to a cyanide buffer for a further 10 s and immediately rinsed in tap water. Tissue sections were then incubated with 0.05 M Tris buffer (pH 7.6) containing 10 mg diaminobenzidine and 5% sucrose for 10 min. Fresh 1% hydrogen peroxide (0.1 ml) was added to the above Tris buffer, and slides were incubated for a further 20 min while observing the development of the stain. Transferring slides to 0.05 M Tris for 10 min stopped the reaction. Sections were counterstained with haematoxylin and mounted. Sections were coded and read in a blind fashion, and counts were expressed as number of cells per millimetre of basement membrane.

2.6. Measurement of cytokines

Cytokines were measured in a subset of mice from each experimental group. Briefly, frozen lung tissue was homogenized using a motorised tissue grinder in 5–10 volumes of ice-cold lysis buffer containing 20 mM Tris pH7.4, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 1% Triton-X 100, and protease inhibitor cocktail tablet (Complete Mini, EDTA free protease inhibitor cocktail, Roche). Samples were centrifuged at 1000 $\times g$ for 5 min at 4 °C. The supernatant was removed and centrifuged for a further 10 min at 12,000 $\times g$ at 4 °C. Samples were aliquoted into 10 ìl and stored at −80 °C. Cytokines were measured using Endogen (Rockford, IL, USA) enzyme-linked immunosorbent assay kits. Results are expressed as picogram per milligram of lung protein.

2.7. Measurement of c-Jun NH_2-terminal kinase activation

Lung tissue was obtained from four animals from each group. Briefly, 50 μg of total lung protein per lane was separated through 8% denaturing polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in the following buffer (Tris 20 mM, pH 7.6, NaCl 140 mM, and 0.1% Tween) and then incubated for 1 h with affinity-purified rabbit polyclonal antibodies anti-nonphosphorylated c-*jun* (Cell Signalling Technology, Beverly, MA) and anti-phosphorylated c-*jun* (P-c-*jun*; Cell Signalling Technology) as markers of JNK activity. We used an equal mix of the antiphosphoserine-63 and -73 antibodies as these sites are important for activation of c-*jun*. The secondary antibody was horseradish-peroxidase-conjugated sheep antimouse or antirabbit (diluted to 1:10,000), and Enhanced Chemiluminescence (Amersham, UK) reagent was used for detection. The bands, which were visualised by autoradiography, were quantified using a densitometer with Grab-It and GelWorks software (UVP, Cambridge, UK).

2.8. Measurement of SP600125 in lung tissue

Lung samples were weighed and homogenized using 80% methanol in water. Samples were extracted by protein precipitation using equal volumes of methanol containing internal standard. Samples were centrifuged, and the supernatant was directly injected for quantitation. A liquid chromatography and mass spectrometry (LC/MS/MS) method was used for quantitation of SP600125 using electrospray in the positive ionization mode and multiple reaction monitoring using a triple quadrupole instrument (Micromass Quattro Ultima, USA). Reverse phase chromatography (Waters, USA) was performed with an isocratic method held at 50% *B* (*B*=5%, *A*+95% acetonitrile, *A*—water with 0.1% formic acid) using a 4.6×50 mm liquid chromatography column (Varian, Polaris, C18-A). Linear regression analysis was used for the calibration of standards.

2.9. Data analysis

Data are presented as means±standard error of mean (S.E.M.). Statistical analysis was performed using a nonparametric analysis of variance (ANOVA) test for multiple comparisons. Nonparametric student's *t*-tests were used to compare differences between two groups. A *P* value less than 0.05 was considered significant.

3. Results

3.1. Bronchial responsiveness

There were no significant differences in the baseline lung resistance values following PBS challenge in the four experimental groups (data not shown). There was a significant increase in bronchial responsiveness to acetylcholine in sensitised allergen-exposed and vehicle-treated mice ($-\log PC_{200}$: 1.71±0.08) compared to sensitised PBS-exposed and vehicle-treated mice ($-\log PC_{200}$: 2.12±0.09; $P<0.01$). Treatment of allergen-exposed mice with SP600125 significantly inhibited the allergen-induced increase in bronchial responsiveness to a level similar to that of PBS-exposed control mice ($-\log PC_{200}$: 2.14±0.09; $P<0.01$). There was no significant change in bronchial responsiveness when SP600125 was administered to sensitised PBS-exposed mice ($-\log PC_{200}$: 2.12±0.99) (Fig. 1).

3.2. Bronchoalveolar lavage fluid

In ovalbumin-sensitised vehicle-treated and ovalbumin-exposed group, there was a significant increase in total cell count ($200.90\pm17.90\times10^3$/ml vs. $70.00\pm9.63\times10^3$/ml) compared to ovalbumin-sensitised vehicle-treated PBS-exposed animals. There was also a significant increase in eosinophils ($84.77\pm11.93\times10^3$/ml vs. $0.01\pm0.01\times10^3$/ml;

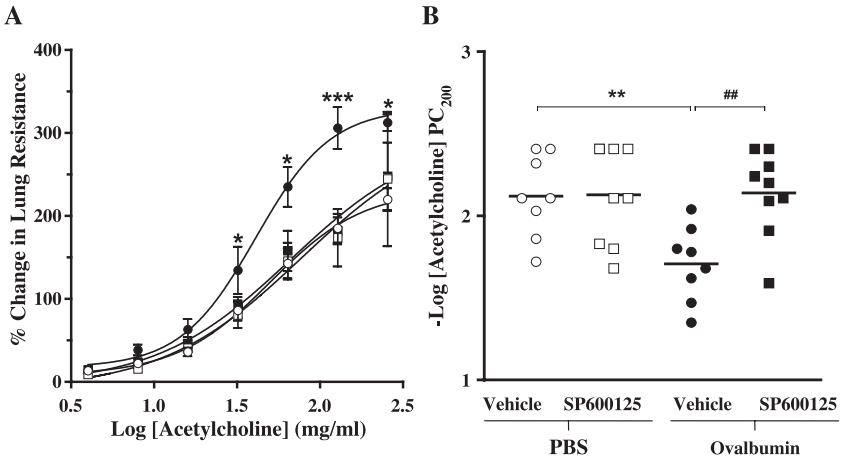

Fig. 1. (A) Mean percentage increase in lung resistance to increasing concentrations of acetylcholine. Four groups of sensitised mice were assessed: vehicle-treated and PBS exposed (○; n=8), SP600125-treated and PBS-exposed (□; n=8), vehicle-treated and ovalbumin-exposed (●; n=8), and SP600125-treated and ovalbumin-exposed (■; n=9). Data is expressed as means±S.E.M. *P<0.05, ***P<0.001 compared to SP600125-treated and ovalbumin-exposed animals. (B) Individual and mean $-\log PC_{200}$ measured 72 h following allergen exposure. Following allergen exposure, vehicle-treated ovalbumin-exposed animals exhibited a significantly lower $-\log PC_{200}$ compared to vehicle-treated PBS-exposed group. **P<0.01. SP600125 inhibited the decrease in the $-\log PC_{200}$ after allergen challenge. (##P<0.01). Data expressed as means±S.E.M.

P<0.001) and lymphocytes (45.91±5.24×10^3/ml vs. 2.72±0.90×10^3/ml; P<0.01). SP600125 significantly reduced the allergen-induced increase in total cell count (P<0.05), eosinophils (P<0.001), and lymphocytes (P<0.01) when compared to the ovalbumin-sensitised vehicle-treated allergen-exposed group (Fig. 2). There was no significant difference between ovalbumin-sensitised SP600125-treated and PBS-exposed group compared to ovalbumin-sensitised vehicle-treated and PBS-exposed mice.

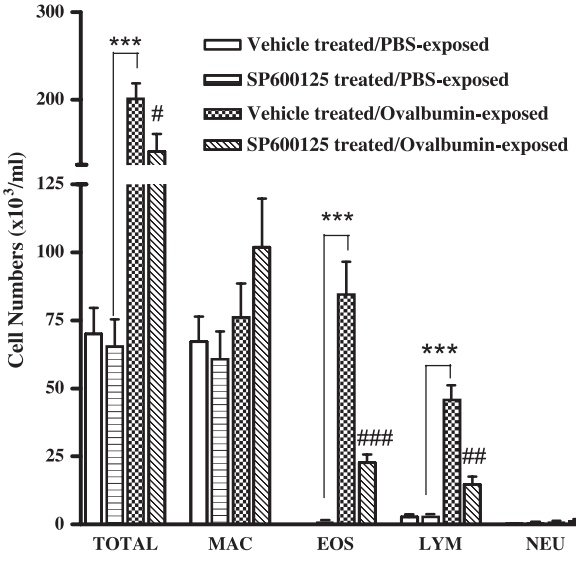

Fig. 2. Mean numbers of total cells (Total), macrophages (Mac), eosinophils (Eos), lymphocytes (Lym), and neutrophils (Neu) in bronchoalveolar lavage fluid. In sensitised vehicle-treated ovalbumin-exposed animals (n=8), there was a significant increase in the total cell count (***P<0.001), eosinophils (***P<0.001), and lymphocytes (***P<0.001) as compared to vehicle-treated ovalbumin-exposed and PBS-exposed group (n=8). SP600125 significantly reduced the allergen-induced increase in total cells (n=9; #P<0.05), eosinophils (###P<0.001), and lymphocytes (##P<0.01). Data shown as means±S.E.M.

3.3. Eosinophilic inflammation

Vehicle-treated allergen-exposed animals showed a significant increase in eosinophil peroxidase positive eosinophils (10.30±2.45 vs. 1.63±0.35 cells/mm basement membrane; P<0.001) when compared to the vehicle-treated PBS-exposed group. There was a significant decrease in the number of eosinophil peroxidase positive cells in animals treated with SP600125 exposed to allergen. There was no significant difference between groups challenged with PBS and treated with either vehicle or SP600125 (Fig. 3).

3.4. Airway smooth muscle hyperplasia

There was a significant increase in the number of airway smooth muscle nuclei in vehicle-treated allergen-exposed animals (8.81±0.93 vs. 6.04±0.47 cells/mm basement membrane) compared to the vehicle-treated and PBS-challenged group. Treatment with SP600125 inhibited the allergen-induced increase in airway smooth muscle cells compared with vehicle-treated animals (4.05±0.54 cells/mm basement membrane). There was no significant difference between vehicle-treated and SP600125-treated PBS-exposed animals (Fig. 4).

3.5. Goblet cell hyperplasia

There was a significant increase in the number of goblet cells per millimetre of epithelial membrane in vehicle-treated

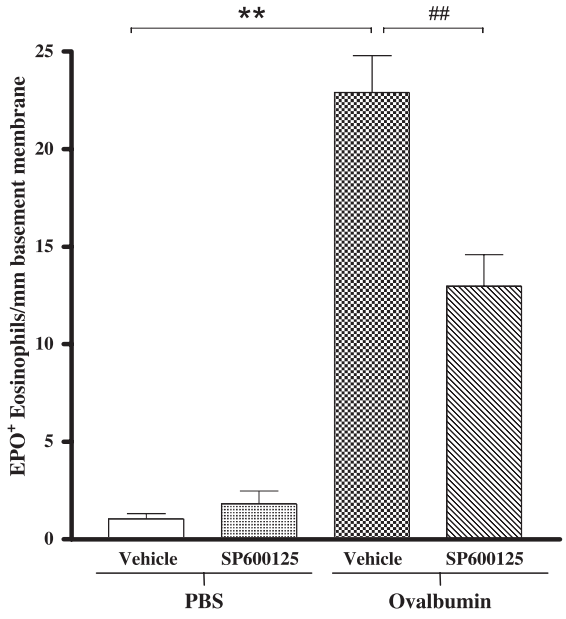

Fig. 3. Mean eosinophils counts in the airway submucosa. Vehicle-treated allergen-exposed mice (n=6) demonstrate increased infiltration of eosinophils (**P<0.001) compared to vehicle-treated PBS-exposed mice (n=5). SP600125 (n=8) caused a decrease in the number of eosinophils compared to the vehicle-treated and allergen-exposed group (##P<0.01). Data shown as means±S.E.M.

allergen-exposed animals (10.67±1.53 vs. 0.70±0.34 goblet cells/mm of epithelial membrane) compared to the vehicle-treated PBS-challenged group. Treatment with SP600125 inhibited the allergen-induced increase in goblet cells compared with vehicle-treated animals (6.99±0.87 goblet cells/mm of epithelial membrane). There was no significant difference between vehicle-treated and SP600125-treated PBS-exposed animals (Fig. 5).

3.6. Cytokine levels in lung homogenates

No significant increase in interleukin-4, interleukin-13, regulated on activation normal T-cell expressed and secreted (RANTES), and tumor necrosis factor-α extracted from lung homogenates were observed following allergen exposure in vehicle-treated animals compared to vehicle-treated and PBS-exposed group. A significant decrease in interleukin-4, interleukin-13, RANTES, and tumor necrosis factor-α was observed following treatment with SP600125 in allergen-exposed animals compared to the vehicle-treated allergen-exposed group (Fig. 6). Furthermore, SP600125 significantly inhibited baseline tumor necrosis factor-α levels in animals exposed to PBS compared to the vehicle-treated PBS-exposed group. No significant decrease in IL-1β was observed following administration of SP600125 in either allergen or PBS-exposed mice compared to vehicle-treated animals.

3.7. c-Jun NH_2-terminal kinase activation

Phosphorylated-c-*jun* (P-c-*jun*) and nonphosphorylated c-*jun* expression were detected in lung tissue from animals treated with vehicle or SP600125 and exposed

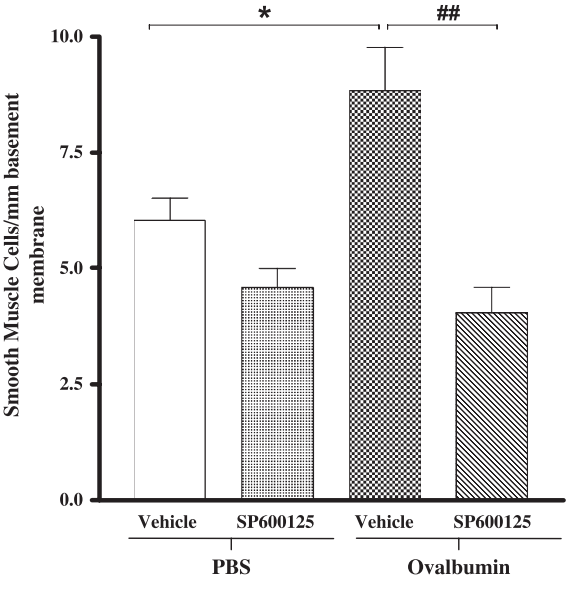

Fig. 4. Mean number of airway smooth muscle cells. An increase in the number of airway smooth muscle cells (*P<0.05) was observed in vehicle-treated allergen-exposed mice (n=6) compared to vehicle-treated PBS-exposed mice (n=8). SP600125 caused a decrease in the number of airway smooth muscle cells in mice challenged with allergen (n=7) compared to the vehicle-treated allergen-exposed group (##P<0.01). Data shown as means±S.E.M.

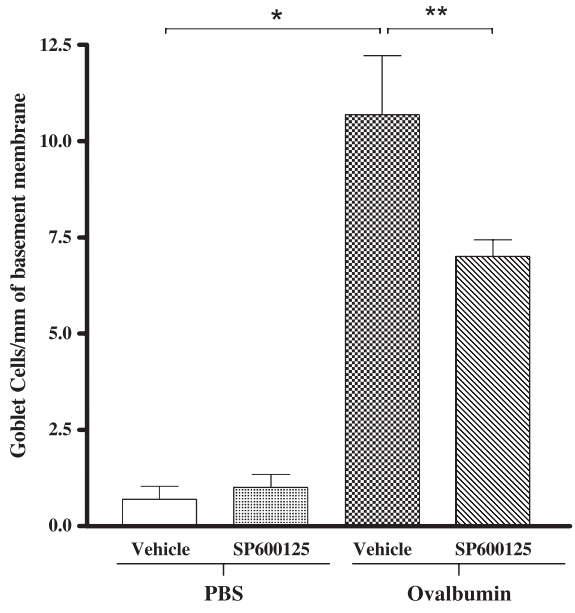

Fig. 5. Mean number of goblet cells. Vehicle-treated allergen-exposed mice (n=6) demonstrated an increase in the number of goblet cells (*P<0.05) compared to vehicle-treated PBS-exposed mice (n=4). SP600125-treated mice exposed to allergen (n=4) demonstrated a significant decrease in the number of smooth muscle cells compared to the vehicle-treated allergen-exposed group (**P<0.01). Data shown as means±S.E.M.

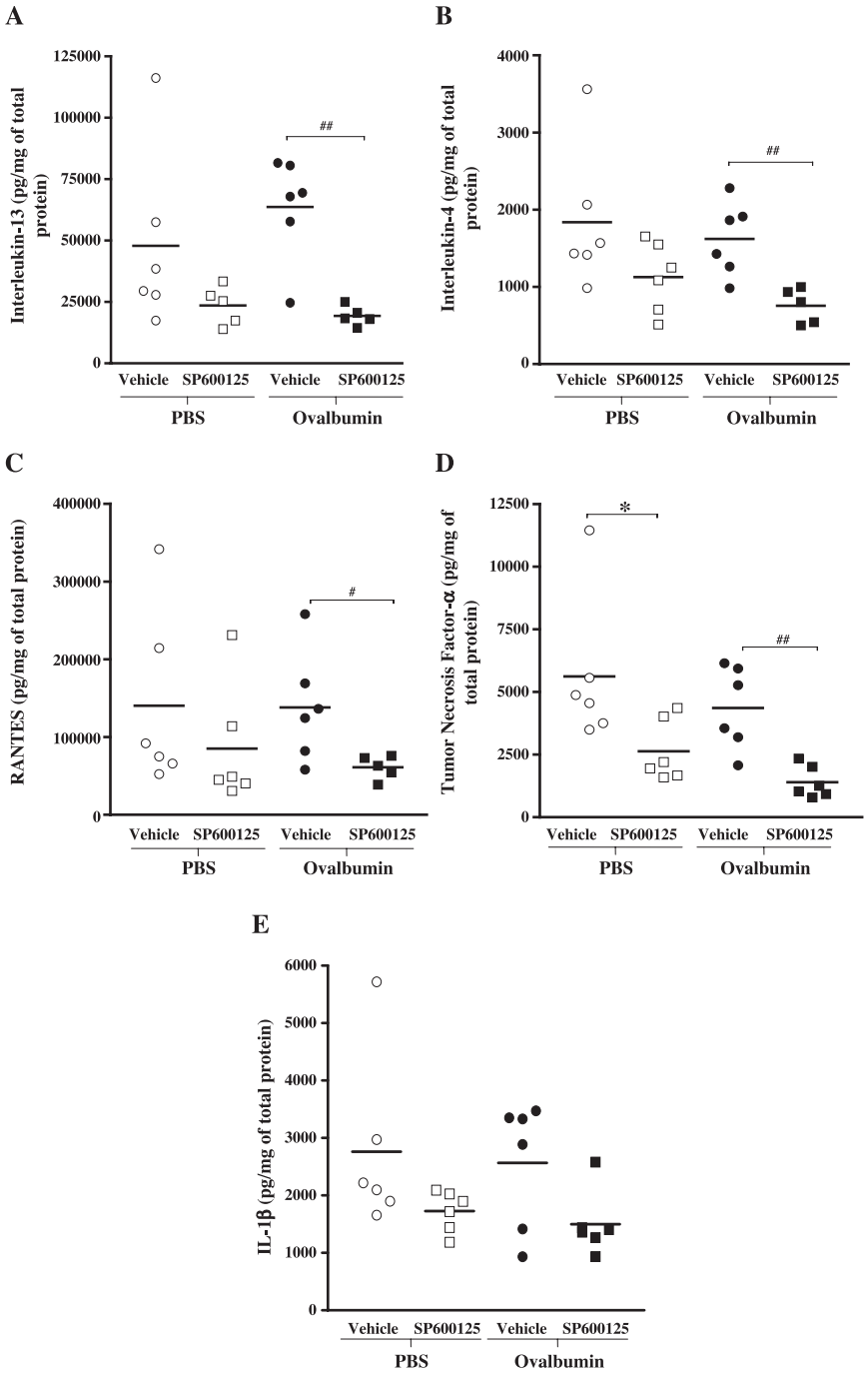

Fig. 6. Interleukin-13 (A), interleukin-4 (B), RANTES (C), tumor necrosis factor-α (D), and interleukin-1β (E) levels in lung homogenates. No significant difference in the above cytokines was observed in vehicle-treated animals exposed to ovalbumin compared to vehicle-treated and PBS-exposed mice. SP600125 significantly inhibited the levels of interleukin-13 (n=5), interleukin-4 (n=5), RANTES (n=5), and tumor necrosis factor-α (n=6; #P<0.05, ##P<0.01) following ovalbumin-exposure. A significant decrease in baseline tumor necrosis factor-α was observed following treatment with SP600125 in animals exposed to PBS (n=6), compared to vehicle-treated PBS-exposed mice (n=6; *P<0.05). No change in interleukin-1β was observed in animals treated with SP600125 and exposed to ovalbumin. Data shown as means±S.E.M.

to either PBS or ovalbumin, as shown by Western blot analysis (Fig. 7). SP600125 significantly inhibited P-c-Jun expression in ovalbumin-exposed animals. SP600125 did not alter the expression of P-c-Jun in PBS-exposed animals.

3.8. SP600125 in lung tissue

The volume of distribution for SP600125 in mouse following i.p. dosing was calculated to be 0.36 l/kg, indicating a partitioning biased to the plasma rather than

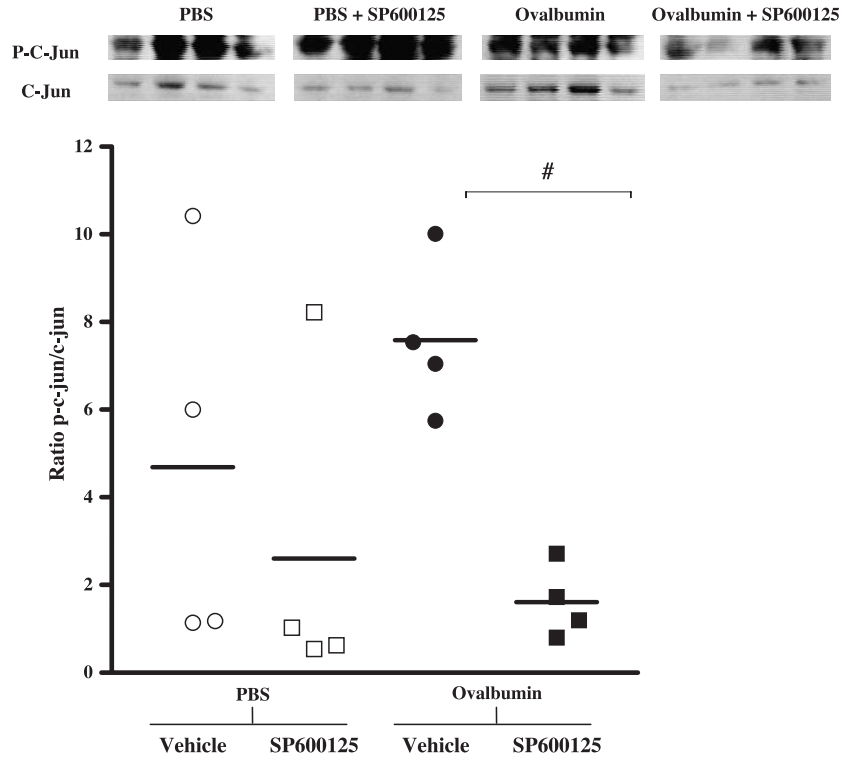

Fig. 7. Western blot analysis of P-c-Jun. P-c-Jun expression was nonsignificantly elevated in vehicle-treated ovalbumin-exposed mice (●; n=4) compared to vehicle-treated PBS-exposed animals (○; n=4). SP600125-treated mice exposed to allergen (■; n=4) demonstrated a significant decrease in P-c-Jun compared to vehicle-treated allergen-exposed animals (#P<0.05). Data shown as means±S.E.M.

tissues (less than 1). Taking into the account this volume of distribution, counterbalanced by the knowledge that lung is highly vascularized, then the values we observed (0.5 and 3 μM) are completely reasonable based on previously observed plasma concentrations of 2–3 μM at 2 h after 30 mg/kg i.p. dosing.

4. Discussion

Using a novel inhibitor of c-Jun NH_2-terminal kinase, SP600125, we evaluated the role of c-Jun NH_2-terminal kinase in a murine model of allergic asthma. Following chronic allergen exposure of sensitised Balb/C mice, we found accumulation of inflammatory cells, an increase in the number of airway smooth muscle cells and goblet cells, and an increase in bronchial responsiveness. Pretreatment with SP600125 attenuated allergen-induced bronchial hyperresponsiveness and significantly inhibited eosinophil and lymphocyte accumulation in bronchoalveolar lavage fluid. This coincided with a decrease in eosinophil peroxidase positive eosinophil accumulation within the bronchial submucosa. In addition, SP600125 inhibited allergen-induced airway goblet cell and airway smooth muscle cell increase. These observations indicate that c-Jun NH_2-terminal kinase activity may be important in the chronic inflammation and airway remodelling process and accompanying bronchial hyperresponsiveness induced by chronic allergen exposure in the mouse. These results are similar to those observed in a rat model of chronic allergen exposure in that airway smooth muscle cell proliferation and allergic cellular inflammation were inhibited, but bronchial hyperresponsiveness was not (Eynott et al., 2003). A decrease in the lung content of interleukin-4, interleukin-13, RANTES, and tumor necrosis factor-α was also observed following administration of SP600125; however, no change in interleukin-1β levels was observed.

SP600125 is a reversible ATP-competitive inhibitor with more than a 20-fold selectivity over a range of kinases and enzymes and has been shown to dose-dependently inhibit the phosphorylation of c-*jun* and the expression of inflammatory genes and to prevent the differentiation of primary human CD4 cell cultures (Bennett et al., 2001). In a mouse model of endotoxin-induced inflammation, pretreatment with SP600125 at 30 mg kg^{-1} significantly inhibited tumor necrosis factor-α serum levels (Bennett et al., 2001), and, therefore, in the current study, this dose was employed. Similar to this study, we found that SP600125 inhibited tumor necrosis factor-α content in the lungs of mice following either PBS or allergen exposure. In addition, SP600125, used at the same dose of 30 mg kg^{-1} in an adjuvant arthritis model in the rat, inhibited joint inflammation by attenuating metalloproteinase expression and joint destruction with suppression of c-Jun NH_2-terminal kinase activation in the synovium (Han et al., 2001). Direct measurement of SP600125 in the lungs of treated rats

revealed levels of the order of 0.5 and 3 μM, which is within the range where this compound is active as an inhibitor of c-*jun* N-terminal kinase activity and also reasonably selective. By measuring the phosphorylation of c-*jun* in rat lung, we found that SP600125 inhibited c-*jun* N-terminal kinase activity following allergen exposure. However, the question of selectivity of SP600125 cannot be entirely confirmed in this in vivo situation.

We used a chronic allergen exposure model rather than a single exposure model because we were particularly interested in the role of the c-Jun NH_2-terminal kinase in the remodelling and chronic inflammatory changes. In our chronic allergen exposure model, we found that levels of eosinophils in bronchoalveolar lavage fluid were significantly reduced by the selective c-Jun NH_2-terminal kinase inhibitor, SP600125. Coincidentally, the amount of lung RANTES at the protein level was also significantly attenuated following treatment with SP600125. RANTES is an important chemoattractant for eosinophil (Venge et al., 1996), monocytes (Becker and Soukup, 1999), and T-cells (Taguchi et al., 1998). RANTES production by bronchial epithelial cells (Kujime et al., 2000) and by human airway smooth cells (Oltmanns et al., 2003) has been demonstrated to be partly regulated by c-Jun NH_2-terminal kinase. Our data therefore implicates a potential role for the c-Jun NH_2-terminal kinase pathway in the recruitment of eosinophils into the airways. However, the effect of SP600125 on eosinophil recruitment was only partial, indicating that other pathways not requiring the c-Jun NH2-terminal kinase are important. We have previously reported that SP600125 significantly inhibited major basic protein positive eosinophils in allergen-exposed Brown Norway rats (Eynott et al., 2003). Recently, it has been reported that the inhibition of pulmonary eosinophilia does not prevent airway hyperresponsiveness in Lewis Rats (Matsubara et al., 1998). In addition, allergen-exposed interleukin-5 transgenic mice exhibiting profound eosinophilia demonstrate no bronchopulmonary hyperreactivity to methacholine or serotonin (Lefort et al., 1996).

In the current study, we also observed a significant reduction in lymphocytes recovered in bronchoalveolar lavage fluid following treatment with SP600125, supporting the role of the c-Jun NH_2-terminal kinase pathway in cellular trafficking of inflammatory cells. The endothelial cell adhesion molecules, such as E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intracellular adhesion molecule-1 (ICAM-1), are dynamically regulated at sites of allergic inflammation, and cytokine-induced E-selectin gene expression can be activated by c-Jun NH_2-terminal kinase. In addition, SP600125 can inhibit ICAM-1 gene expression (Bennett et al., 2001). Therefore, cell migration can be reduced through SP600125 inhibition of endothelial cell adhesion.

T helper type 2 cytokines, such as interleukin-4, interleukin-5, and interleukin-13 appear to underlie the inflammation of asthma. The overexpression of these cytokines has been demonstrated in the airways of asthmatics (Robinson et al., 1992). We observed that SP600125 inhibited protein levels of interleukin-4 and interleukin-13 in lung tissue, demonstrating a role for c-Jun NH_2-terminal kinase in regulating Th-2 cytokine production. It has also been shown that antigen-induced interleukin-13 production from rat basophilic leukaemia (RBL-2H3) cells can be inhibited by SP600125 (Hirasawa et al., 2003). Interleukin-4 and interleukin-13 promoters contain an activator protein-1 binding site directly regulated by c-Jun NH_2-terminal kinase (Rooney et al., 1995; Buitkamp et al., 1999); therefore, c-Jun NH_2-terminal kinase may in turn partially control interleukin-4 and interleukin-13 production. Furthermore, a significant reduction in the baseline levels of the proinflammatory cytokine, tumor necrosis factor-α, was observed following pretreatment with SP600125 in control mice. Analysis of the tumor necrosis factor-α gene promoter has identified DNA binding elements for transcription factors associated with c-Jun NH_2-terminal kinase, namely, c-*jun*; in addition, it has been determined that SP600125 can inhibit tumor necrosis factor-α gene expression in a dose-dependent manner in monocytic cells stimulated with LPS (Bennett et al., 2001).

Our data indicates that c-Jun NH_2-terminal kinase may be involved in aspects of airway remodelling as treatment with SP600125 led to inhibition of allergen-induced airway smooth muscle and goblet cell hyperplasia. This is likely to be a direct effect on the proliferation of these structural cells. The induction of airway smooth muscle hyperplasia appears to be rapid but was likely initiated from the first of the seven exposures to ovalbumin aerosol, i.e., 23 days prior to collection of lungs. Inhibition of c-Jun NH_2-terminal kinase by the use of antisense has been shown to inhibit epidermal-growth-factor-induced proliferation of A549 lung carcinoma cells (Bost et al., 1997). c-Jun NH_2-terminal kinase activation also plays an important part in vascular endothelial-growth-factor-induced G1/S progression and cell proliferation (Pedram et al., 1998). Treatment of primary rat aortic cells with SP600125 also led to suppression of vascular smooth muscle cell proliferation in vitro (Kavurma and Khachigian, 2003). In addition, we have shown that SP600125 inhibits airway smooth muscle and epithelial cell proliferation induced by chronic allergen exposure in a rat model, as measured by the incorporation of bromodeoxyuridine (Eynott et al., 2003). Furthermore, interleukin-1β-induced rat airway smooth muscle cell proliferation in vitro is also inhibited by SP600125 (Zhai et al., 2004).

The inflammatory and structural changes in the airways of asthmatics have been associated with bronchial hyperresponsiveness (Pare et al., 1997). The functional consequences of airway wall thickening due to increased airway smooth muscle mass have resulted in increased airway resistance leading to bronchial hyperresponsiveness. Furthermore, the increase in airway smooth muscle mass may be a direct result of the chronic inflammation observed. Eosinophils and other inflammatory cells are a

rich source of growth factors for airway smooth muscle proliferation. Our study provides evidence that chronic inflammation and airway remodelling are associated with bronchial hyperresponsiveness in the mouse. However, we have found that, while SP600125 inhibits chronic inflammation and airway remodelling in the chronic rat model, there is no effect on bronchial hyperresponsiveness (Eynott et al., 2003). This may be due to species differences, but this indicates that bronchial hyperresponsiveness may not be directly related to chronic inflammation and airway remodelling changes. In addition, this differential species-specific effect also suggests that different mechanisms may underlie the bronchial hyperresponsiveness in different species. Thus, we cannot extrapolate this finding in mouse of the potential importance of the c-Jun NH_2-terminal kinase pathway in human asthma.

Our study supports a role for the c-Jun NH_2-terminal kinase pathway in chronic inflammation, airway wall remodelling, and bronchial hyperresponsiveness. However, since we are not able to completely exclude other potential effects of SP600125, other pathways may also be involved. Inhibition of c-Jun NH_2-terminal kinase may provide a novel target therapy for concomitantly controlling many features of asthma.

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