



Importance of p38 mitogen-activated protein kinase pathway in allergic airway remodelling and bronchial hyperresponsiveness

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Abstract

p38 mitogen-activated protein kinase (MAPK) plays an important role in the activation of inflammatory cells and in the proliferation of airway structural cells. We investigated the role of p38 MAPK by using a selective inhibitor of p38 α and β isoforms, SD282, in a chronic model of 15 ovalbumin exposures in sensitised mice using two doses (30 and 90 mg/kg). Allergen exposure induced bronchial hyperresponsiveness to methacholine as measured by the concentration of methacholine needed to increase pulmonary resistance by 200% (PC₂₀₀), eosinophilia in bronchoalveolar lavage fluid and increase in airway smooth muscle area and goblet cell hyperplasia. In addition, p38 MAPK activity as measured by phosphorylated p38 expression on Western blots was increased after allergen challenge, which was suppressed by SD282 at both doses. SD282 inhibited bronchial hyperresponsiveness, but had no effect on eosinophils in bronchoalveolar lavage fluid. It also reduced airway smooth muscle and goblet cell hyperplasia, but had no effect on serum immunoglobulin E. p38 MAPK is involved in the pathogenesis of bronchial hyperresponsiveness but not in eosinophilic inflammation or the allergic response; however, remodelling features such as airway smooth muscle or goblet cell hyperplasia are regulated through p38 MAPK. Furthermore, bronchial hyperresponsiveness induced by chronic allergen exposure may be related to the development of airway wall remodelling.

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1. Introduction

The mitogen-activated protein kinases (MAPKs) are an important activating pathway that integrate and process various extracellular signals that could mediate gene expression, cell differentiation, growth and apoptosis. Three distinct MAPK pathways have been described in mammalian cells: the extracellular signal-regulated kinases (ERKs) pathway, the c-Jun amino terminal kinase (JNK) pathway and the p38 MAPK pathway (Davis, 1993). While the ERKs are activated usually by mitogenic and proliferative stimuli, the JNKs and p38 MAPKs respond to envi-

ronmental stress, including ultraviolet light, heat, osmotic shock and inflammatory cytokines. Of the four isoforms of p38 MAPK, p38 α , p38 β , p38 γ and p38 δ , p38 α is the most characterized and is likely to be the most relevant kinase involved in inflammatory responses (Kumar et al., 2003). p38 MAPK is activated by dual phosphorylation on Threonine180 and Tyrosine182 by an upstream MAPK kinase (MAPKK) termed MAP2K6, which is in turn activated by several MAPKKKs, which are activated by a wide variety of stimuli. The p38 α MAPK pathway is crucial in inflammation, cell death and cell proliferation (Zarubin and Han, 2005), and has been implicated in many cellular events relevant to asthma pathophysiology.

Thus, the features central to the pathogenesis of the asthma such as eosinophil activation and cytokine release (Adachi et al., 2000), eosinophil apoptosis (Kankaanranta et al., 1999),

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interleukin (IL)-5 and IL-13 synthesis by Th-2 cells (Chen et al., 2000a; Mori et al., 1999) and the expression of low-affinity immunoglobulin (Ig)E receptor (Marshall et al., 1998) have been associated with p38 MAPK activation. Furthermore, evidence implicating a role for p38 MAPK activation in leukocyte recruitment has been reported since inhibition of p38 MAPK attenuated lipopolysaccharide and tumour necrosis factor (TNF)- α stimulated intercellular adhesion molecule (ICAM)-1 regulation in human pulmonary microvascular endothelial cells (Tamura et al., 1998). Eotaxin-induced degranulation and chemotaxis of eosinophils is partly dependent on p38 MAPK activation (Kampen et al., 2000). Structural cells within the airways have also been associated with p38 MAPK activation. IL-6 production in fibroblasts induced by contact with mast cells is dependent on p38 MAPK activation (Fitzgerald et al., 2004) and in addition, transforming growth factor (TGF)- β -induced p38 MAPK activation mediates apoptosis of human bronchial epithelial cells (Pelaia et al., 2003).

The role of p38 MAPK has been previously investigated in a single allergen exposure models in sensitised rodents. SB239063, a p38 MAPK inhibitor, reduced the number of allergen-induced eosinophils in bronchoalveolar lavage fluid from sensitised mice (Underwood et al., 2000), while in another study in sensitised rats, another inhibitor, SB203580, had no effects on ovalbumin-induced eosinophilia (Escott et al., 2000). Other investigators have reported that using antisense oligonucleotides to p38 MAPK, there was an inhibition of bronchial hyperresponsiveness, eosinophils and goblet cell hyperplasia (Duan et al., 2005). However, little is known about the role of p38 MAPK in airway wall remodelling changes observed in a chronic model of allergen exposure, particularly in terms of airway smooth muscle changes. We have therefore used an orally active p38 MAPK inhibitor, SD282 (Sweitzer et al., 2004), in order to investigate the role of the p38 MAPK in a model of chronic allergen exposure in BALB/c mice.

2. Methods

2.1. Animals, sensitisation procedures and allergen exposure

The protocols were approved by the Imperial College Bio-Sciences group and performed under a licence from the Home Office, UK. 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 Inc., Hazelton, PA, USA) and were sensitised on day 0 by intraperitoneal injection containing 10 μ g ovalbumin (Chicken Egg Ovalbumin, Grade V) and 2 mg aluminium hydroxide (BDH, Dorset, UK) in 0.2 ml sterile phosphate-buffered saline. On Day 14, the mice were given an intraperitoneal booster injection of the same allergen/aluminium hydroxide mix.

Seven days after the second sensitisation, mice were challenged with aerosolised ovalbumin (50 mg/ml in phosphate-buffered saline) or phosphate-buffered saline alone. Ovalbumin aerosol exposure was performed by placing animals in a 6.5 l Plexiglas chamber, connected to an ultrasonic nebulizer (DeVilbiss, UK). The aerosol was circulated through the chamber using a ventilator (Harvard Apparatus Ltd.) generating an aerosol mist. Animals were exposed to allergen for 20 min per day, for five

consecutive days, over a period of three weeks. Seventy two hours after the last aerosol challenge, bronchial responsiveness to acetylcholine was measured and bronchoalveolar lavage fluid and lung tissues were obtained.

Four groups of mice sensitised to ovalbumin were studied:

- (1) Vehicle-treated, phosphate-buffered saline-exposed animals: Mice were treated with vehicle one hour prior and six to eight hours following phosphate-buffered saline exposure.
- (2) Vehicle-treated, ovalbumin-exposed animals: Mice were treated with vehicle one hour prior and six to eight hours following ovalbumin exposure.
- (3) SD282-treated (30 mg/kg), ovalbumin-exposed animals: Mice were treated with SD-282 (30 mg/kg) one hour prior and six to eight hours following ovalbumin exposure.
- (4) SD282-treated (90 mg/kg) and ovalbumin-exposed: Mice were treated with SD-282 (90 mg/kg) one hour prior and six to eight hours following ovalbumin exposure.

2.2. SD-282

SD-282 (Scios Inc., Fremont, CA, USA) is an indole-5-carboxamide selective p38 α MAPK inhibitor demonstrating a 14.3-fold greater potency for p38 α compared to p38 β (IC_{50} 1.6 $\pm$ 0.3 nM versus 23 $\pm$ 2.3 nM) (Sweitzer et al., 2004). It does not inhibit p38 δ and p38 γ ; in addition, there are no direct inhibitory effects on COX-1 and COX-2 enzymes (Svensson et al., 2003a,b), and no detectable effects against human MAPK including JNK1, JNK2, JNK3, ERK1 and ERK2. MAPK activated protein kinase 2, p38 activating kinases MKK3 and MKK6 are not inhibited *in vitro* up to a concentration of 50 μ M (Lim et al., 2004; Medicherla et al., 2006). SD-282 was dissolved in a 1% solution of PEG-400 and administered to mice by oral gavage. Vehicle control animals received 1% solution of PEG-400. All doses were administered one h prior to and six to eight hours following allergen/phosphate-buffered saline exposure. A small pharmacokinetic study performed in male mice reveals that serum levels of SD-282 was maximal within 30 min at 25 μ M for an oral dosing of 30 mg/kg and at $\sim$ 65 μ M for 90 mg/kg. The levels fell to zero and $\sim$ 15 μ M respectively at 6 h.

2.3. Measurement of bronchial responsiveness

Mice were anaesthetised with an intraperitoneal injection of anaesthetic solution containing midazolam (Roche Products Ltd., 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, with a tidal volume of 250 μ l. 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, Herts, UK). 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 pulmonary resistance, increasing concentrations of acetylcholine chloride (acetylcholine) (Sigma, Dorset, UK) (4–256 mg/ml) were administered by aerosol for 45 s at each concentration with an ultrasonic nebulizer, and pulmonary 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 (in order to ensure a constant volume history prior to each concentration). The maximum pulmonary resistance values were expressed as percentage change from baseline pulmonary resistance measured after phosphate-buffered saline aerosol. The concentration of acetylcholine needed to increase pulmonary resistance by 200% above phosphate-buffered saline baseline (PC₂₀₀) 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 response, mice were killed using an overdose of pentobarbitone (60 mg/kg intraperitoneally, Sagatal; May and Baker Ltd., UK). The trachea was catheterised. Six separate 0.5 ml aliquots of phosphate-buffered saline were injected into the tracheal cannula and collected as the bronchoalveolar lavage sample. The lavage fluid was centrifuged at 500 $\times g$ 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 μ l of the cell suspension to 90 μ l of Kimura stain. Differential cell counts were prepared on cytospins (Shandon, UK) by adding 50–200 μ l of the cell suspension and centrifuging at 200 $\times g$ for 5 min. Cytospins were stained with May–Grunwald 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 phosphate-buffered saline/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 tissue 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 (6 μ m) in a cryostat (Bright OTF Cryostat, Bright Instruments Co. Ltd., Huntingdon) alongside the main intrapulmonary bronchus and mounted on glass slides pre-coated with poly-L-lysine for improved binding. Sections were stored at –20 °C before being used.

2.6. Haematoxylin and eosin staining airway smooth muscle cells

Sections were fixed in 10% formalin buffered saline for 10 min, and washed in distilled water for 2 min. Sections were

then transferred to 20% Haematoxylin (BDH, UK) for 30 s and immediately washed in tap water for 2–3 min. Sections were then transferred to 1% eosin (BDH, UK) for 10 s and immediately washed in tap water for 2–3 min (changing water every min). Finally, sections were dehydrated through graded alcohols (70%, 90%, 100%), cleared through xylene (BDH, UK) and mounted with DPX. Sections were coded and read; counts were expressed as number of cells per mm of basement membrane.

2.7. Quantification of airway smooth muscle cells

Quantification of airway smooth muscle cells were performed using a Zeiss Light microscope (Welwyn Garden City, Herts, UK). Using an image analysis system (KS 300 model, Imaging Associates Ltd., Thame, UK), the whole airway was visualised with an $\times 5$ objective lens. The internal perimeter was measured as the basement membrane length. The airway was then visualised under an $\times 40$ objective lens and the number of elongated airway smooth muscle cell nuclei stained with haematoxylin were counted within 100 μ m of the basement membrane. Counts were expressed as the number of airway smooth muscle cells per mm of basement membrane.

2.8. Periodic Acid Schiff (PAS) and Alcian Blue stain 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, UK) 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 mm of epithelial membrane.

2.9. Total serum IgE assay

A 96-well plate was coated with LO-ME-3 anti-mouse IgE in bicarbonate coating buffer. The plate was washed three times and 50 μ l of samples and standards were added to the plate. A standard curve was prepared using purified mouse IgE, 400 ng/ml being the highest concentration. Samples were incubated overnight at 40 °C. The plate was washed three times and 50 μ l of OX-2-biotin was added to the plate. Following a two hour incubation at room temperature, the plate was washed three times and extravidin–alkaline phosphatase was added. Samples were incubated for one hour and *p*-nitrophenylphosphate in diethanolamine buffer was added. The plate was read at 405 nm. Total IgE is expressed as ng/ml.

2.10. Western blotting

Frozen lung tissues were homogenised and total protein was extracted using a motorised tissue grinder. All samples were kept on ice in radioimmunoprecipitation buffer (phosphate-buffered saline containing 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1% Igepal, 200 μ M Na_3VO_4 , and protease inhibitor cocktail) and homogenised until the solution turned clear. Samples were then sonicated on ice five times to clear any remaining debris. Following sonication, samples were centrifuged at 8000 $\times g$, at 4 °C for 10 min and aliquots of supernatant were stored at –80 °C.

Protein concentration in lung supernatant was measured using a protein assay kit (Pierce Chemical Company, Rockford, USA). At least 30 μ g of protein per lane was loaded onto 4–12% polyacrylamide precast gel (Novex, Invitrogen, Paisley, UK). The gel was then run and transferred to nitrocellulose membrane. Membranes were blocked for 45 min at room temperature in tris-buffered saline containing 0.05% (w/v) Tween-20%, 5% (w/v) non-fat dried milk. The membrane was then incubated with an anti-phosphorylated threonine and tyrosine residues of p38 (New England Biolabs Inc., US; 1:1000) overnight at 4 °C. Following incubation, the membrane was washed in tris-buffered saline/Tween-20 and incubated with a secondary antibody, a goat anti-rabbit conjugated to horseradish peroxidase for 1 h at room temperature. The membrane was developed using enhanced chemiluminescence (LumiGLO; KPL Europe, Guildford, UK) as recommended by the manufacturer. Bands were visualised by autoradiography and were quantified using a densitometer with Grad-It and Gel Work software (UVP, Cambridge, UK). The membrane was then stripped, and re probed with a phosphorylation state-independent anti-p38 antibody. Densitometric data for the phosphorylated band were expressed as a ration of the total non-phosphorylated band.

2.11. Data analysis

Data are presented as mean $\pm$ S.E.M. Statistical analysis was performed using a non-parametric analysis of variance test for multiple comparisons. Non-parametric student *t*-tests were used to compare differences between two groups. A *P* value of less than 0.05 was considered significant.

3. Results

3.1. p38 MAPK activation

Because our target was inhibition of p38 MAPK activation in the lungs, we first determined whether p38 MAPK was activated after chronic allergen exposure and whether SD-282 had inhibitory effects on MAPK activation. We detected p38 in lung tissues from animals treated with vehicle or 30 mg/kg or 90 mg/kg of SD-282 and exposed to either phosphate-buffered saline or ovalbumin, as shown by Western blot analysis (Fig. 1). Both 30 mg/kg and 90 mg/kg SD-282 significantly inhibited phosphorylated p38 MAPK expression in ovalbumin-exposed animals.

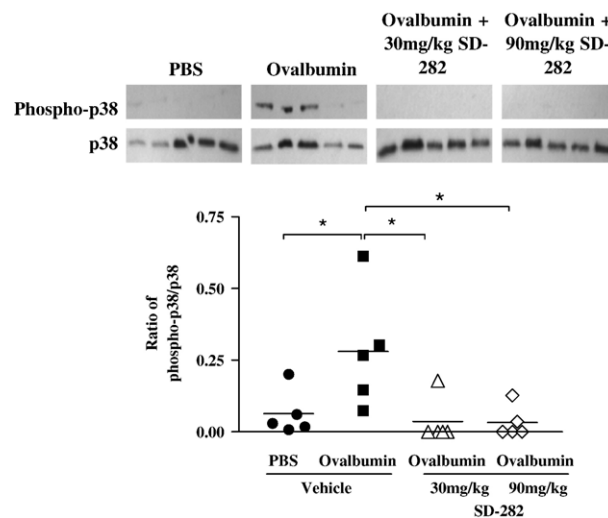


Fig. 1. Western blot analysis of phosphorylated-p38 MAPK. phospho-p38 MAPK expression was significantly elevated in vehicle-treated, ovalbumin-exposed mice (■) (*n*=5) compared to vehicle-treated, phosphate-buffered saline-exposed animals (●) (*n*=5). SD-282 (30 mg/kg (Δ) and 90 mg/kg (◇)) treated mice (*n*=5) exposed to allergen demonstrated a significant decrease in p-p38 MAPK compared to vehicle-treated, allergen-exposed animals (*; *P*<0.05). Data shown as mean $\pm$ S.E.M.

3.2. Bronchial responsiveness

There was no significant difference in baseline lung resistance between the four experimental groups. Following allergen exposure, vehicle-treated animals demonstrated a significant increase in bronchial responsiveness ($-\log \text{PC}_{200} -1.8 \pm 0.1$ versus -2.4 ± 0.0 , *P*<0.01), compared to phosphate-buffered saline-exposed mice treated with vehicle. Following treatment with SD-282 30 mg/kg, a non-significant reduction in bronchial responsiveness was observed in allergen-exposed mice compared to allergen-exposed vehicle treated animals. Treatment with SD-282 90 mg/kg induced a significant reduction in bronchial responsiveness ($-\log \text{PC}_{200} -2.1 \pm 0.1$ versus -1.8 ± 0.1 ; *P*<0.05) (Fig. 2A and B).

3.3. Bronchoalveolar lavage fluid

Vehicle-treated mice exposed to allergen demonstrated a significant increase in total cells (313.4 ± 28.7 versus $82.8 \pm 9.7 \times 10^3$ cells/ml; *P*<0.0001) compared to vehicle-treated phosphate-buffered saline-exposed mice. The increase in total cells comprised of a significant increase in lymphocytes (95.1 ± 7.4 versus $5.1 \pm 0.6 \times 10^3$ cells/ml; *P*<0.0001), and eosinophils (135.1 ± 16.9 versus $0.2 \pm 0.0 \times 10^3$ cells/ml; *P*<0.0001). Treatment with 30 mg/kg and 90 mg/kg SD-282 in allergen-challenged mice did not affect total cell numbers compared to vehicle-treated allergen-exposed animals. However, a reduction in lymphocytes was observed in allergen-exposed mice treated with 30 mg/kg SD-282 (63.0 ± 8.3 versus $95.1 \pm 7.4 \times 10^3$ cells/ml; *P*<0.05) compared to vehicle-treated allergen challenged animals. However, this was not noted at 90 mg/kg SD-282. SD-282 had no effect on eosinophil numbers in bronchoalveolar lavage fluid (Fig. 3).

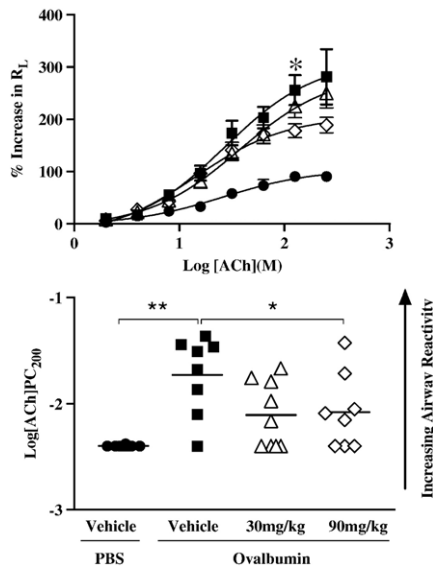


Fig. 2. Bronchial responsiveness to acetylcholine. Panel A: Mean percentage increase in lung resistance to increasing concentrations of acetylcholine. Four groups of sensitized mice were assessed: vehicle-treated and PBS exposed (●) ($n=8$), vehicle-treated and ovalbumin-exposed (■) ($n=8$), SD-282 (30 mg/kg) treated and ovalbumin-exposed (△) ($n=9$), SD-282 (90 mg/kg) treated and ovalbumin-exposed (◇) ($n=8$). * $P<0.05$, allergen-exposed animals treated with SD-282 (90 mg/kg) were significantly less responsive compared to vehicle-treated ovalbumin-exposed mice. Panel B: Individual and mean $-\log PC_{200}$. * $P<0.05$ and ** $P<0.01$. Data expressed as mean $\pm$ S.E.M.

3.4. Airway smooth muscle

Allergen-exposure in vehicle-treated mice induced a significant increase in the number of airway smooth muscle cells (13.6 ± 1.6 versus 9.5 ± 0.3 cells/mm of basement membrane, $P<0.05$) compared to vehicle-treated phosphate-buffered saline-challenged mice. Treatment with SD-282 dose-dependently inhibited the allergen-induced increase in airway smooth muscle cells. Following treatment with SD-282 (30 mg/kg) a non-significant reduction in airway smooth muscle cells was observed in allergen-exposed mice (10.8 ± 0.8 versus 13.6 ± 1.6 cells/mm of basement membrane, $P<0.07$) compared to

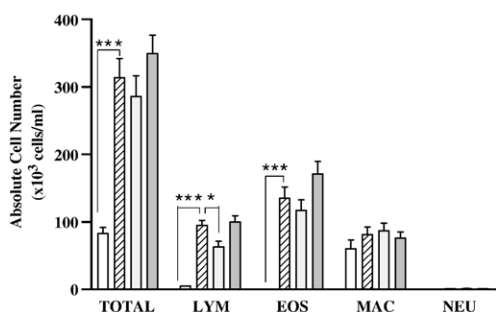


Fig. 3. Mean numbers of total cells (Total), macrophages (Mac), eosinophils (Eos), lymphocytes (Lym) and neutrophils (Neu) in bronchoalveolar lavage fluid. Vehicle treated, ovalbumin-exposed animals (■) ($n=8$) demonstrated a significant increase in total cells (***; $P<0.0001$), eosinophils (***; $P<0.0001$), and lymphocytes (***; $P<0.001$) compared to, vehicle treated, phosphate-buffered saline-exposed group (●) ($n=8$). 30 mg/kg SD-282 at 30 mg/kg (△) but not at 90 mg/kg (◇) ($n=8$) significantly reduced allergen-induced increase in lymphocytes (*; $P<0.05$). Data shown as mean $\pm$ S.E.M.

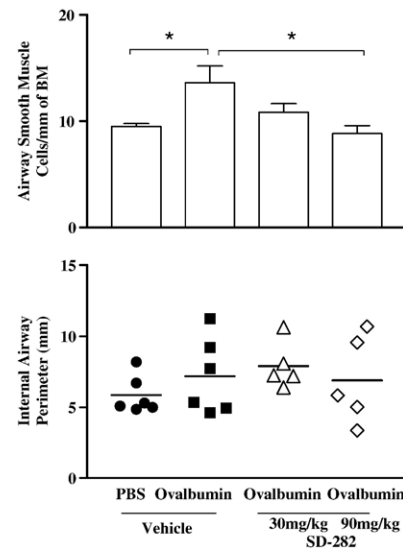


Fig. 4. Panel A: An increase in the number of airway smooth muscle cells (*; $p<0.05$) was observed in vehicle-treated, allergen-exposed mice ($n=6$; dashed bars) compared to vehicle-treated and phosphate-buffered saline-exposed mice ($n=6$; open bars). SD-282 (30 and 90 mg/kg; stippled and grey bars) induced a dose-dependent decrease in the number of airway smooth muscle cells in mice challenged with allergen, compared to the vehicle treated, allergen-exposed group (*; $P<0.05$). Panel B: No significant difference in airway size was observed between experimental groups. ● = vehicle treated, PBS-exposed mice. ■ = Vehicle-treated, allergen-exposed mice. △ = 30 mg/kg SD-282-treated, ovalbumin-exposed. ◇ = 90 mg/kg SD-282-treated, ovalbumin-exposed. Data shown as mean $\pm$ S.E.M.

vehicle-treated animals exposed to ovalbumin. SD-282 (90 mg/kg) significantly reduced the allergen induced increase in airway smooth muscle cells (8.8 ± 0.7 versus 13.6 ± 1.6 cells/mm, $P<0.05$) compared to vehicle-treated animals (Fig. 4A). Airway perimeter was measured in all groups to determine the size of the airways measured. Four airways were measured per mouse lung to obtain an average airway size. There was no significant difference in mean airway size between groups (Fig. 4B).

3.5. Goblet cell hyperplasia

Allergen-exposure in vehicle-treated animals evoked a significant increase in goblet cells (31.2 ± 2.3 versus 5.0 ± 1.7 goblet

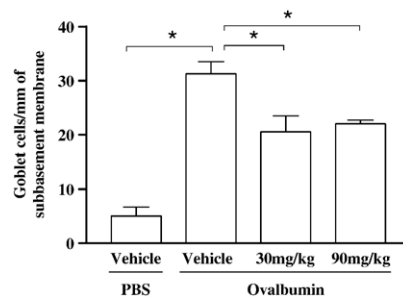


Fig. 5. Vehicle-treated, allergen-exposed mice ($n=4$) demonstrated an increase in the number of goblet cells (*; $p<0.05$), compared to vehicle treated, phosphate-buffered saline-exposed mice ($n=4$). SD-282-treated mice at both doses (30 mg/kg; $n=5$ and 90 mg/kg; $n=4$) exposed to allergen demonstrated a significant decrease in the number of goblet cells, compared to the vehicle-treated, allergen-exposed group (*; $P<0.05$). Data shown as mean $\pm$ S.E.M.

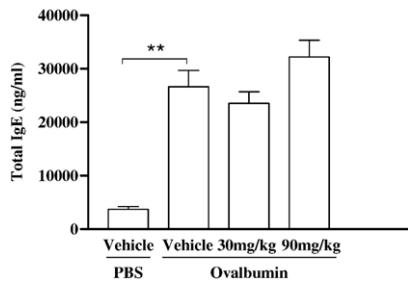


Fig. 6. Sensitisation and allergen exposure induced a significant increase in total serum IgE in vehicle-treated animals (** $P < 0.01$). SD-282 had no effect on total serum IgE. Data are presented as mean $\pm$ S.E.M.

cells per mm of basement membrane, $P < 0.05$) compared to vehicle-treated phosphate-buffered saline-challenged animals. Following administration of SD-282 (30 mg/kg), a significant reduction in allergen-induced goblet cells were observed (20.5 ± 2.9 versus 31.2 ± 2.3 goblet cells per mm of basement membrane, $P < 0.05$) and similarly SD-282 (90 mg/kg) also inhibited goblet cell metaplasia (22.0 ± 0.7 versus 31.2 ± 2.3 goblet cells per mm of basement membrane, $P < 0.05$) compared to vehicle-treated mice exposed to allergen. There was no significant difference in the number of allergen-induced goblet cells in mice treated with either 30 mg/kg or 90 mg/kg SD-282 (20.5 ± 2.9 versus 22.0 ± 0.7 goblet cells per mm of basement membrane) (Fig. 5).

3.6. Serum IgE

Following sensitisation and allergen exposure, a significant increase in total serum IgE was observed in ovalbumin-sensitised, vehicle-treated allergen exposed mice ($26,630 \pm 3045$ versus 3711 ± 511 ng/ml; $P < 0.01$) compared to ovalbumin-sensitised, vehicle-treated phosphate-buffered saline-challenged animals. Treatment with 30 mg/kg and 90 mg/kg SD-282 did not affect total serum IgE levels in allergen-exposed mice (Fig. 6).

4. Discussion

We have investigated the role of p38 MAPK pathway in a chronic allergen exposure murine model of allergic inflammation and airway wall remodelling, using a selective p38 α MAPK inhibitor, SD-282. Following chronic allergen exposure of sensitised BALB/c mice, we found that treatment with SD-282 significantly attenuated allergen-induced increase in airway smooth muscle hyperplasia, goblet cell hyperplasia, and allergen-induced bronchial hyperresponsiveness. SD-282 had a small inhibitory effect on the number of bronchoalveolar lavage fluid lymphocytes but had no effect on bronchoalveolar lavage fluid eosinophil counts. Serum IgE was unaffected by SD-282. These observations indicate that the p38 MAPK activation may be important in the airway remodelling process that could underlie chronic allergen-induced bronchial hyperresponsiveness.

SD-282 is a small molecule that selectively inhibits the phosphorylation of p38 α MAPK (Sweitzer et al., 2004). SD-282 is 14.3-fold more selective in inhibiting the activity of the p38 α isoform compared to p38 β and has no detectable activity

against other human MAP kinases including JNK1, JNK2, JNK3, ERK1 and ERK2 (Sweitzer et al., 2004; Medicherla et al., 2006). In our study, allergen-exposure in BALB/c mice induced the activation of p38 MAPK in lung tissue as measured by the amount of phosphorylated p38 by Western blotting and SD-282 clearly inhibited p38 activation at both 30 mg/kg and 90 mg/kg. Therefore, SD-282 inhibited p38 MAPK activation following chronic allergen exposure. This inhibition of p38 MAPK activity by SD-282 could result in inhibition of inflammatory cytokine release as has been shown in human peripheral blood mononuclear cells ex-vivo (Lim et al., 2004). One potential reason why we saw inhibitory effects at the higher dose of 90 mg/kg is that much higher serum levels of SD-282 were maintained for up to 2 h with this dose compared to the 30 mg/kg dose, as we have shown in a small pharmacokinetic study in mice. It is also likely that selectivity for p38 α MAPK inhibition was maintained at the 90 mg/kg dosing that achieved maximum plasma levels of 50 μ M (Sweitzer et al., 2004; Medicherla et al., 2006), but this remains to be confirmed in the *in vivo* situation.

In terms of inhibition of the various parameters we observed in our chronic allergen mouse model, we found that many of the inhibitory effects of SD-282 were already present at the dose of 30 mg/kg. The inhibitory effect of SD-282 at that dose on bronchial hyperresponsiveness was not statistically significant because of the distribution of the PC₂₀₀ values, but was only significant at the higher dose of 90 mg/kg despite the mean values at these two concentrations are similar. Inhibition of goblet cell hyperplasia was observed at both doses without a clear-cut dose-response.

Increased numbers of CD4⁺ T-lymphocytes have been detected in the airways of asthmatic subjects (Bentley et al., 1993) and have been associated with the induction of bronchial hyperresponsiveness in allergen-exposed mice (Gavett et al., 1994). Th-2 cytokines such as IL-4, 5 and 13 secreted by CD4⁺ Th-2 cells are known to play an essential role in the pathogenesis of allergic inflammation and contribute towards bronchial hyperresponsiveness (Kuperman et al., 2002; Rankin et al., 1996), eosinophilic inflammation (Lefort et al., 1996) and IgE synthesis (Maggi et al., 1989). Activation of p38 kinase in Th-2 cells contributes towards increased production of IL-5 and IL-13 (Chen et al., 2000a). Furthermore, the inhibition of p38 MAPK using an anti-p38 monoclonal antibody can prevent the release IL-5 and IL-13 from endotoxin-stimulated mast cells (Masuda et al., 2002). The p38 MAPK pathway may also be important in the differentiation of eosinophils and in apoptosis of eosinophils (Adachi et al., 2000; Kankaanranta et al., 1999; Underwood et al., 2000). However, in our study, SD-282 had only little effects in reducing the number of bronchoalveolar lavage lymphocytes only at the lower dose of SD282 (30 mg/kg), without any effect on bronchoalveolar lavage eosinophils; in addition, total serum IgE was not reduced. These results indicate that there was likely to be little effect of p38 MAPK inhibition on the expression of the Th2 cytokines, IL-4 and IL-5 that are important in eosinophilic and IgE production. The small effect on lymphocyte counts may be related to blockage of vascular cell adhesion molecule-1 expression and T-cell adhesion through inhibition of p38 MAPK (Ju et al., 2002).

In contrast to our data, Duan et al. (2005) and Underwood et al. (2000) have demonstrated that the inhibition of p38 MAPK using anti-sense oligonucleotides or a small molecule inhibitor respectively inhibited the development of eosinophilic inflammation following allergen-exposure in mice (Duan et al., 2005; Underwood et al., 2000). However, in a study in sensitised rat, another p38 MAPK inhibitor had no effect on eosinophilia. The difference between these studies to ours may be related to the use of repeated allergen exposures in our study.

The most interesting data of our study is that p38 MAPK is involved in aspects of airway wall remodelling as treatment with SD-282 inhibited airway smooth muscle hyperplasia and goblet cell hyperplasia. SD-282 has been shown to inhibit isoproterenol-induced collagen type I and fibronectin gene expression in a model of myocardial infarction (Li et al., 2004). Collagen type I and fibronectin are both components of the extracellular matrix, and can enhance platelet-derived growth factor-induced human airway smooth muscle proliferation (Nguyen et al., 2005). Zhai et al. have demonstrated that IL-1 β induced proliferation of airway smooth muscle cells from rats partly through the activation of the p38 MAPK pathway (Zhai et al., 2004). In asthma, airway smooth muscle also retains the capability of producing pro-inflammatory cytokines and chemokines that can perpetuate the inflammatory response (Chung, 2000). The p38 MAPK pathway has been shown to be involved in IL-1 β -induced release of monocyte chemoattractant protein (MCP)-1 and eotaxin (Wuys et al., 2003). Therefore, in the context of the airway smooth muscle cell, p38 MAPK may be involved in its proliferation and in the induction of chemokines.

In addition to a reduction in allergen-induced airway smooth muscle hyperplasia, a significant decrease in mucus-secreting goblet cells was observed following treatment with SD-282 compared to wild-type mice treated with vehicle treatment alone. Mucin gene expression can be induced by Th-2 cytokines such as IL-4, and IL-13 (Dabbagh et al., 1999; Chu et al., 2004). IL-13 induced goblet cell hyperplasia and MUC5A protein expression in human bronchial epithelial cells, and this could be inhibited by the p38 MAPK inhibitor, SB 202190 (Atherton et al., 2003). Moreover, IL-1 β and TNF- α induced MUC5A gene expression in normal human nasal epithelial cells is also abolished following treatment with SB 203580, another p38 MAPK inhibitor (Song et al., 2003).

In a previous study, we have examined a JNK inhibitor, SP600125, and found that similar to the p38 MAPK inhibitor that there was inhibition of chronic allergen-induced goblet cell hyperplasia and airway smooth muscle hyperplasia; however, in addition, there was inhibition of eosinophilia and bronchial hyperresponsiveness (Nath et al., 2005). This indicates that other MAPK may be involved in allergic inflammation and remodelling. It would be interesting to observe whether inhibition of these two members of the MAPK family would have additive or synergistic effects.

Although SD-282 did not have an effect on eosinophil recruitment in the present study, the inhibition of p38 dose dependently inhibited bronchial hyperresponsiveness, suggesting that eosinophils may not be important in the induction of bronchial hyperresponsiveness. The dissociation of airway eosino-

philia and bronchial hyperresponsiveness has been reported previously (Matsubara et al., 1998; Eynott et al., 2003; Leckie et al., 2000).

We have shown that the inhibition of p38 MAPK pathway following treatment with SD-282 attenuates allergen-induced bronchial hyperresponsiveness which coincided with the inhibition of airway smooth muscle hyperplasia and goblet cell hyperplasia but not eosinophilic inflammation.

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