Effect of p38 MAPK inhibition on corticosteroid suppression of cytokine release in severe asthma

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\textit{Running title:} p38 MAPK inhibition and corticosteroid effects

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ABSTRACT (207w)

Background
Patients with severe asthma respond less well to corticosteroids than those with non-severe asthma. Increased p38 mitogen-activated protein kinase (MAPK) activation in alveolar macrophages (AMs) from severe asthma patients has been associated with a reduced inhibition of cytokine release by dexamethasone.

Question of the study
We determined whether p38 MAPK inhibitors would modulate corticosteroid suppression of cytokine release from AMs and peripheral blood mononuclear cells (PBMC).

Methods
PBMCs were isolated from venous blood and AMs by bronchoalveolar lavage in severe and non-severe asthma patients. PBMCs and AMs were exposed to lipopolysaccharide (LPS) with and without the p38 MAPK inhibitor, SD282, or dexamethasone. We determined the concentration-dependent effects of another p38 MAPK inhibitor, GW-A, on dexamethasone-induced inhibition of IL-8 release from PBMCs. Cytokines were assayed using an ELISA-based method.

Results
SD282 (10^{-7}M), with dexamethasone (10^{-6}M), caused a greater inhibition of release of IL-1β, IL-6, MIP-1α and IL-10 than with dexamethasone alone in AMs from severe and non-severe asthma. At 10^{-9} and 10^{-10}M GW-A, that had no direct effects, increased the inhibitory activity of dexamethasone (10^{-8} and 10^{-9}M) on LPS-induced IL-8 release in PBMCs from severe asthma. Similar results were observed with IL-6 release.

Answer to the question.
Corticosteroid insensitivity in severe asthma patients may be improved by inhibitors of p38 MAPK.

**Key words**

Alveolar macrophages, corticosteroid-resistant asthma, cytokines, p38 mitogen-activated protein kinase, severe asthma.
Introduction

Most asthma patients are well-controlled on inhaled corticosteroid therapy (ICS), but a small proportion experience continuing symptoms. These patients labelled as having severe asthma consume a more significant proportion of medical resources in terms of drugs, admissions to hospital or use of emergency services, and time off work or school [1]. Definitions and the clinical features of recently-described cohorts attest to the persistent loss of control of asthma despite the optimal use of asthma medication. Such patients demonstrate a poor response to the therapeutic effects of corticosteroids. Peripheral blood mononuclear cells (PBMCs) and alveolar macrophages (AMs) from patients with severe asthma are less sensitive to inhibition by dexamethasone in terms of the stimulated release of pro-inflammatory cytokines, when compared with cells from well-controlled non-severe asthma patients[2;3].

The mechanisms underlying this poor suppressive response to corticosteroids in severe asthma are unclear, but many have been proposed from in vitro studies in cells[4]. We observed that AMs from patients with severe asthma demonstrated a greater degree of activation of p38 mitogen-activated protein kinase (MAPK) [2]. p38 MAPK is a family of serine-threonine kinase that act on a variety of substrates including transcription factors, such as NF-κB and AP-1 and has been implicated in inflammation, cell proliferation and cell death relevant to asthma pathophysiology[5]. We hypothesised that activation of p38 MAPK could be linked to the reduced inhibition of cytokine release by corticosteroids since part of the action of corticosteroids may relate to inhibition of MAPK activation. In addition, p38 MAPK activation has been previously shown to induce corticosteroid insensitivity in peripheral blood mononuclear cells through
phosphorylation of the glucocorticoid receptor[6], indicating one potential mechanism of corticosteroid insensitivity.

Selective inhibitors of p38 MAPK, mainly the α isoform, have been developed. In seeking to implicate a role for p38 MAPK activation in corticosteroid sensitivity, we examined whether inhibitors of p38 MAPK could improve the ability of dexamethasone in suppressing cytokine release in AMs and PBMCs from patients with severe asthma.
METHODS

Study participants

Asthma patients with either bronchodilator responses to albuterol of $\geq 12\%$ of baseline $\text{FEV}_1$ or with a methacholine $\text{PC}_{20}$ of $<16\text{mg/ml}$ (Table 1) were recruited from the Royal Brompton Severe Asthma Clinic. Current and ex-smokers of $>5$ pack-years were excluded. Severe asthmatics were defined according to the ATS criteria[7]. Non-severe asthmatic patients used $0$-$2,000\mu\text{g}$ of inhaled beclomethasone-equivalent per day with control of their asthma. The protocols were approved by the local Ethics Committee. All volunteers gave written informed consent.

Isolation of Alveolar Macrophages

Fibreoptic bronchoscopy was performed with intravenous midazolam and alfentanil. Broncho-alveolar lavage (BAL) was performed in the right middle lobe with $0.9\%\text{NaCl}$ solution. Macrophages ($5 \times 10^5$/well) were purified by adhesion to plastic wells for 4 hours and then exposed for 18 hours to LPS ($10\mu\text{g/ml}$) in the presence or absence of dexamethasone ($10^{-6}\text{M}$) or of a selective p38 MAPK inhibitor, SD282 ($10^{-7}\text{M}$; Scios Inc, Freemont, CA) [8;9] or both.

Isolation of PBMCs

Venous blood ($80\text{ml}$) was diluted 1:1 with Hanks buffered saline solution (HBSS) and layered on Ficoll-Hypaque-Plus. Following centrifugation, PBMCs were re-suspended in culture media, plated ($7.5 \times 10^5\text{cells/well}$) and stimulated with LPS($10\mu\text{g/ml}$) with or without dexamethasone ($10^{-6}\text{M}$) or SD282 ($10^{-7}\text{M}$) or both. Supernatants at 18 hours were analysed for MIP-1$\alpha$, IL-1$\beta$, IL-6 and IL-10.
LPS-stimulated PBMCs were also used to examine the effect of GW-A($10^{-12}$-$10^{-6}$M), a p38 MAPK inhibitor from GlaxoSmithKline, or dexamethasone ($10^{-12}$-$10^{-6}$M) or of the combination of GW-A and dexamethasone on IL-8 release.

**p38 MAPK phosphorylation**

To determine p38 MAPK activity, PBMCs were stimulated with LPS overnight in the presence or absence of dexamethasone ($10^{-8}$ M) and/or GW-A ($10^{-9}$ M). The contents of each well were stored in Beadlyte cell lysis buffer B (Beadlyte®, Upstate Technology, NY) at -70ºC for later assay for phosphorylated and total p38 MAPK using microsphere beads coated with antibodies to P-p38 and total p38 using the Beadlyte® protocol.

**Measurement of cytokine release**

The cytokines MIP-1α, IL-1β, IL-6 and IL-10 were assayed simultaneously using microsphere beads (Beadlyte®) coated with capture antibodies. Biotinylated reporter antibodies were used to bind the microsphere bead-cytokine complexes. Finally, a fluorophore, streptavidin-phycoerythrin was added to bind the biotinylated reporter and the fluorescent signal measured in a laser spectrophotometer (Luminex Corporation, Austin, TX). Microsphere beads for each cytokine emitted a unique ratio of two other fluorophores. IL-8 was measured using ELISA.

**Data analysis**

Results were expressed as mean ± SEM. Cytokine release induced by LPS with or without dexamethasone or p38 MAPK inhibitor was calculated by subtraction of
baseline release. The release of each cytokine following dexamethasone + LPS was calculated as a percentage of cytokine release following LPS stimulation alone. Corticosteroid sensitivity between severe asthma and non-severe asthma patients was compared using the Mann-Whitney U test. The effect of SD282 on the suppressive effect of dexamethasone was analysed by Wilcoxon paired t-test. Concentration-dependent responses were examined using one-way-ANOVA (Kruskal-Wallis test) followed by a Dunn's Multiple Comparison test. p<0.05 was taken as significant.
RESULTS

Corticosteroid insensitivity in AMs and PBMCs

Baseline and LPS-stimulated release of IL-1β, IL-6, MIP-1α and IL-10 from both AMs and PBMCs did not significantly differ between patients with severe asthma and patients with non-severe asthma. There was less inhibition of cytokine release from AMs in the severe asthma group compared with that in the non-severe group for IL-1β, IL-6 and MIP-1α with the percentage of cytokine release after LPS and dexamethasone to that after LPS alone being 71± 8 vs 26 ±7, p<0.01; 52 ± 9 vs 20 ± 5, p<0.05; 70 ± 9 vs 28 ± 7, p<0.01), respectively. There was no significant difference regarding IL-10 (12 ± 4 vs 30 ±10) (Fig 1A).

For PBMCs, there was a non-significant trend for less suppression by dexamethasone in patients with severe asthma compared with non-severe asthma: IL-1β (46 ± 19 vs 27 ± 8); IL-6 (35 ± 8 vs 22 ±6); MIP-1α (48 ±14 vs 45 ± 19); IL-10 (68 ± 10 vs 42 ±9) (Fig 1B).

Effect of SD282 on dexamethasone inhibition of cytokine release

In AMs from non-severe asthma, the suppressive effect of dexamethasone was relatively greater than that of SD282, and there was more inhibition of IL-6 and MIP-1α with the combination, with a trend towards greater suppression for IL-1β (Fig 2A). In AMs from severe asthma, SD282 alone caused only a small degree of suppression of LPS-induced IL-1β, IL-6, MIP-1α and IL−10 release, being similar in magnitude to that seen with dexamethasone alone (Fig 2B). Combined SD282 and dexamethasone caused a greater inhibition of cytokine release compared with dexamethasone alone for the four cytokines.
In PBMCs, the inhibitory effect of SD282 was greater than that observed in AMs. Although there were significant differences between the effect of dexamethasone and that of dexamethasone and SD282 for the release of all four cytokines, with less effect on the release of MIP-1α, from PBMCs of non-severe asthma patients, there was no evidence of additivity in the presence of both dexamethasone and SD282 (Fig 3A). Similar observations were made in PBMCs from severe asthma patients (Fig 3B).

**Effect of dexamethasone and GW-A on p38 activation and IL-8 release from PBMCs**

To determine whether GW-A ($10^{-9}$M) demonstrates inhibitory effects on p38 MAPK activity, the ratio of phosphorylated p38 to total p38 was measured at 24 hours. GW-A ($10^{-9}$M) alone or in combination with dexamethasone ($10^{-8}$M) attenuated p38 activity induced by LPS stimulation (Figure 4).

In order to determine whether there was additivity or synergy between dexamethasone and a p38 MAPK inhibitor, we measured the inhibitory effect of dexamethasone and GW-A alone on LPS-induced IL-8 release from PBMCs from severe asthmatics. A maximal inhibition of $51.2 \pm 4.01$ % release was achieved at $10^{-6}$ M dexamethasone with an IC$_{50}$ of $3.9 \times 10^{-7}$M, while GW-A caused maximal suppression of $51.6 \pm 7.1$% at $10^{-6}$M with an IC$_{50}$ of $3.7 \times 10^{-7}$M.

The effect of dexamethasone at $10^{-6}$M (Fig 5A) in suppressing IL-8 release was improved in the presence of GW-A ($10^{-10}$-$10^{-6}$M) (p<0.0001, one-way-ANOVA) with a maximal suppression of $89.6 \pm 2.6$ % with GW-A at $10^{-6}$ and $10^{-7}$M, compared with $51.9 \pm 4.0$% suppression with dexamethasone alone (p<0.001). At GW-A $10^{-9}$M and $10^{-10}$M, which alone had an inhibitory effect of
less than 3 %, IL-8 suppression by dexamethasone (10^{-6}M) was increased to 76.8 ± 3.5 % (p<0.05) and 70.1 ± 2.0 % (p<0.01), respectively (Fig 5B).

At dexamethasone (10^{-8}M), the inhibitory effect was also improved in the presence of GW-A (p< 0.001, one-way-ANOVA), with maximal suppression with GW-A (10^{-6}M) being 73.4 ± 4.5 % as compared with 31.3 ± 3.8 % with dexamethasone alone. Similar effects were observed with dexamethasone at a concentration of 10^{-9} M, in combination with GW-A (Fig 5A). We obtained similar results when examining the release of IL-6 (Fig 6).
DISCUSSION

We have previously shown impaired corticosteroid sensitivity in AMs and PBMCs from patients with severe asthma and increased activation of p38 MAPK in AMs of severe asthmatics [2;3]. We now demonstrate that a low concentration of a p38 MAPK inhibitor, SD282, that had little effect on IL-1β, IL-6 and MIP-1α release improved the inhibitory activity of dexamethasone in AMs from severe asthma patients. This effect was not observed in PBMCs from patients with severe asthma because the concentration of SD282 chosen had a major suppressive effect on cytokine release. To investigate more closely any interaction between p38 MAPK inhibition and corticosteroids, we studied a wider range of concentrations of another p38 MAPK inhibitor, GW-A, and showed an enhancement of the suppressive effects of dexamethasone in PBMCs from patients with severe asthma at concentrations of GW-A that had no effect on IL-8 release. For example, the maximal suppression of IL-8 release obtained with the highest concentration of dexamethasone (10^-6M), was also achieved with a 100-fold reduction in dexamethasone concentration (10^-8M) with addition of GW-A (10^-9M). The enhancement in the inhibitory effects of dexamethasone appears to be synergistic because the suppression by dexamethasone (10^-9M) by GW-A (10^-9M) alone were 28% and 3%, respectively, whereas the suppression observed when added in combination was increased to 59%.

It is of interest that there was also additivity in the effects of SD282 and dexamethasone in AMs from patients with non-severe asthma in suppressing the release of IL-1β, IL-6 and MIP-1α, albeit to a lesser extent, even though the degree of inhibition by dexamethasone alone was substantial. However, this was not seen with IL-10 release in AMs from non-severe asthma patients, but was
present in AMs from severe asthma patients. Therefore, overcoming corticosteroid insensitivity by using p38 MAPK inhibitors can be demonstrated in severe asthmatics. Our data is supported by a recent study that reported that a p38 MAPK inhibitor in combination with dexamethasone caused a greater suppression of gene expression induced by LPS in monocyte-derived macrophages or AMs [10].

The p38 MAPK inhibitor, SD282, is an indole-5-carboxamide selective p38α MAPK inhibitor demonstrating a 14.3-fold greater potency for p38α compared with p38β [8;9]. No detectable effect on other closely-related kinases such as p38δ, p38γ, jun-N-terminal kinase and p38 activating kinases at concentrations up to 50 mM in human PBMCs has been observed [8], and therefore these effects are likely to result from the selective inhibition of the p38α MAPK isoform. GW-A is another p38 MAPK inhibitor that is currently in clinical development [11]. We have shown that concentrations of GW-A (10^-9 and 10^-10M) that had no effect on cytokine release, still demonstrated significant inhibition of p38 MAPK activity in peripheral blood monocytes exposed to LPS. This suggests that p38 MAPK activation is associated with corticosteroid insensitivity.

Phosphorylation of p38 MAPK was measured as an indicator of the efficacy of GW-A, rather than one of its downstream targets based on studies which have demonstrated that p38 inhibitors can prevent phosphorylation of p38α in vitro [12;13]. Additional studies have demonstrated that p38α can auto-phosphorylate [14] and trans-phosphorylate(19). Finally, SB203508, another p38 MAPK inhibitor related to GW-A, inhibits the enzymatic activity of both activated and unactivated forms of p38α [15].
A role for p38 MAPK activation in severe asthma has been suggested by the observation that macrophages from such patients demonstrate increased p38 MAPK activation when exposed to LPS [2]. Previous studies have indicated a role for p38 MAPK activation in murine asthma models. Thus, in a chronic allergen model, suppression of p38α MAPK by an inhibitor or by antisense oligonucleotides attenuated ovalbumin-induced bronchial hyperreactivity, eosinophilia, goblet cell hyperplasia, airway smooth muscle hypertrophy and bronchial hyperresponsiveness, through a reduction of Th2-cytokines associated with allergic inflammation [16;17]. Another potential effect of p38 inhibition is the reversal of corticosteroid insensitivity that is present in severe asthma [3]. We have previously demonstrated that IL-2- and IL-4-mediated corticosteroid insensitivity of peripheral blood mononuclear cells can be reversed by inhibition of p38 MAPK activity [6]. This insensitivity was reflected by a reduction in corticosteroid ligand binding affinity due to phosphorylation of the GR, that could be reversed by a p38 MAPK inhibitor [6], or by an indirect effect on the ligand binding domain of GR [18]. On the other hand, p38 MAPK activation may also lead to phosphorylation and phosphoacetylation of histones in the promoter regions of NF-κB dependent genes such as those activated by LPS resulting in enhanced recruitment of the transcription factor NF-κB [19;20]. This is consistent with previous observations that histone deacetylase (HDAC) activity is reduced in PBMCs and alveolar macrophages of asthmatic patients [3;21].

p38 MAPK activation may be involved in the stabilisation and increased translation of pro-inflammatory cytokine mRNA, dependent on the conserved AU-
rich elements in the 3'-UTR region [22]. Of the proinflammatory cytokine mRNAs that can be stabilised by p38 MAPK activation in monocyte and macrophage cell lines, IL-1β, IL-6, IL-8, and MIP-1α [23-25] are the same cytokines that were less inhibited by dexamethasone in alveolar macrophages from severe asthma patients, and where the extent of inhibition by dexamethasone was correlated with the degree of p38 MAPK activation [3]. These potential downstream effects of p38 MAPK do not lead to enhanced release of cytokines from the alveolar macrophages but to a reduction in the effectiveness of corticosteroids. Although this study has focused on p38 MAPK activity in this study, activation of other members of the MAPK family such as c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase ERK [26;27] have also been implicated in corticosteroid insensitivity. Whether there are interactions between the parallel downstream pathways to influence corticosteroid sensitivity deserves further investigation. In previous studies, we have also shown the reduced induction of MAPK phosphatase-1 (MKP-1) by dexamethasone in AMs from patients with severe asthma [2]. This suggests another potential mechanism for increased activation of p38 MAPK activity.

The effect of dexamethasone in inhibiting IL-10 release induced by LPS from AMs and PBMCs may seem contradictory when induction of IL-10 release by corticosteroids has been shown in ex vivo studies of alveolar macrophages or blood monocytes from patients who have been treated with either inhaled corticosteroids [28] or systemic methylprednisolone [29]. However, direct incubation of monocytes or AMs stimulated by LPS with dexamethasone led to an inhibition of IL-10 as we [2;3] and others [29] have shown. p38 MAPK inhibition also reduced IL-10 release from PBMCs or AMs, as confirmed recently
in monocytes [30]. We found that the combination of dexamethasone and GW-A caused an increase in inhibition of IL-10 release, particularly in PBMCs and AMs from patients with severe asthma.

Patients with severe asthma need effective new medications that will improve their asthma control. Our study points to a novel approach to the treatment of severe asthma. A p38 MAPK inhibitor may be used as an anti-inflammatory agent and has been shown to be effective in this way in asthma models [16;17]. We have used this inhibitor to demonstrate its capacity to reverse corticosteroid insensitivity. Immunosuppressive drugs such as cyclosporin A and methotrexate have been administered in patients with steroid-dependent asthma to lower maintenance oral corticosteroid dosage, while allowing the control of asthma to remain unchanged [31;32], but these drugs have not proven to be useful. A p38 MAPK inhibitor could be effective in reversing corticosteroid insensitivity at lower doses than those needed to inhibit inflammation with a lesser risk of side-effects, but will need to be used concomitantly with corticosteroids.
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FIGURE LEGENDS

Figure 1. Concentrations of cytokines in cell culture supernatants of alveolar macrophage (A & B) and peripheral blood mononuclear cells (A & C) from patients with non-severe and severe asthma, at baseline (A) and stimulated with LPS (10 μg/ml) in the presence and absence of dexamethasone (10^{-6}M). There were no significant differences in baseline and stimulated levels of IL-1β, IL-6, MIP-1α and IL-10 between patients with severe and non-severe asthma. There was a significant decrease in release of cytokines in the presence of dexamethasone for both cell types and both patient groups.

Figure 2. Inhibition of LPS-induced cytokine (IL-1β, IL-6, MIP-1α, IL-10) release from alveolar macrophages of a group of 6 non-severe (Panel A) and 6 severe asthmatics (Panel B) by p38 MAPK inhibitor (SD282; 10^{-7}M), dexamethasone (10^{-6}M), or both. * p<0.05.

Figure 3. Inhibition of LPS-induced cytokine (IL-1β, IL-6, MIP-1α) release from PBMCs of a group of 6 non-severe (Panel A) and 6 severe asthmatics (Panel B) by p38 MAPK inhibitor (SD282; 10^{-7}M), dexamethasone (10^{-6}M), or both. * p<0.05, ** p<0.01.

Figure 4. Inhibition of LPS-induced p38 phosphorylation in peripheral blood mononuclear cells from severe asthmatic patients (n=3) by the p38 inhibitor, GW-A (553; 10^{-9}M) in the presence or absence of dexamethasone (Dex; 10^{-8}M). ** p < 0.01, * p < 0.05.
Figure 5. Inhibition of LPS-induced IL-8 release from peripheral blood mononuclear cells by the p38 inhibitor, GW-A (n=8), in the presence of different concentrations of dexamethasone (Panel A), and by dexamethasone in the presence of different concentrations of GW-A (Panel B). *** p < 0.001, ** p < 0.01, * p < 0.05 compared to dexamethasone, at the given concentration, alone.

Figure 6. Inhibition of LPS-induced IL-6 release from peripheral blood mononuclear cells by the p38 inhibitor, GW-A (n=5), in the presence of different concentrations of dexamethasone (Panel A), and by dexamethasone in the presence of different concentrations of GW-A (Panel B). *** p < 0.001, ** p < 0.01, compared to dexamethasone, at the given concentration, alone.
Table 1.
A. Characteristics of non-severe and severe asthma subjects in the SD282 study

| BAL                | Non-severe asthma | Severe asthma |
|--------------------|-------------------|---------------|
| Gender (F:M)       | 3:3               | 5:1           |
| Age (years)        | 32.8 ± 3.88       | 47±2.67       |
| FEV₁ (% predicted) | 89.8 ± 3.28       | 52.8± 7.73*** |
| Bronchodilator response (%)<sup>1</sup> | 9.1 ± 2.24       | 23.3 ±3.97    |
| Log PC<sub>20</sub> (mg/ml) | 0.52 ± 0.15       | -0.60± 0.18   |
| Prednisolone (mg/day) | 0               | 12.5 ± 4.33** |
| BDP equivalent (μg/day) | 33.3 ± 33.3       | 2800 ± 744*** |

**BAL Cells**

| Total count (x10<sup>6</sup>) | 5.85 ± 0.81 | 5.05 ± 0.49 |
| Macrophage (%)                | 94.55 ± 1.37| 92.9 ± 1.63 |
| Neutrophils (%)               | 1.8 ± 0.46  | 3.32 ± 1.57*|
| Eosinophils (%)               | 1.13 ± 0.51 | 1.25 ± 0.82 |
| Lymphocytes (%)               | 2.54 ± 0.43 | 2.53 ± 1.12 |

**PBMC**

| Gender (F:M)       | 2:4               | 5:1           |
| Age (years)        | 36.7 ± 3.61       | 43.2 ± 5.71   |
| FEV₁ (% predicted) | 88.2 ± 3.32       | 55.2± 8.19*** |
| Bronchodilator response (%)<sup>1</sup> | 8.7 ± 2.0        | 21.3 ± 9.4   |
| Log PC<sub>20</sub> (mg/ml) | 0.0 ± 0.28       | 0.1 ± 0.09    |
| Prednisolone (mg/day) | 0               | 8.3 ± 4.01*  |
| BDP equivalent (μg/day) | 333.3 ± 151      | 3200 ± 722*** |

B. Characteristics of severe asthma subjects in the GW-A study.

| PBMC                | Severe asthma |
|---------------------|---------------|
| Gender (F:M)        | 6:2           |
| Age (years)         | 43.2 ± 5.71   |
| FEV₁ (% predicted)  | 68.8 ± 8.19   |
| Bronchodilator response (%)<sup>1</sup> | 20.1 ± 8.4   |
| Log PC<sub>20</sub> (mg/ml) | -1.67 ± 0.21  |
| Prednisolone (mg/day) | 16.25 ± 8    |
| BDP equivalent (μg/day) | 1725 ± 146   |

*p<0.05; **p≤0.01; ***p≤0.001 compared to non-severe asthma
Abbreviations: BAL: bronchoalveolar lavage; BDP: beclomethasone dipropionate; F=female; M=male; FEV<sub>1</sub>=forced expiratory volume in one second; FVC= forced vital capacity; PC<sub>20</sub>= provocative concentration of methacholine causing a 20% fall in FEV<sub>1</sub>; PBMC= Peripheral blood mononuclear cells. Values represent mean ± SEM. \(^1\)Measured as per cent increase over baseline FEV<sub>1</sub> after 400 μg albuterol aerosol.
1. (2000) Proceedings of the ATS Workshop on Refractory Asthma . Current Understanding, Recommendations, and Unanswered Questions. *Am. J.Respir.Crit. Care Med.* **162**, 2341-2351.

2. Adcock, I. M., Ford, P. A., Bhavsar P., Ahmed T., and Chung K.F. Steroid resistance in asthma: mechanisms and treatment options. Curr Allergy Asthma Rep 8(2), 171-178. 2008.

3. Adcock,I.M., Chung,K.F., Caramori,G., & Ito,K. (2006) Kinase inhibitors and airway inflammation. *European Journal of Pharmacology* **533**, 118-132.

4. Bhavsar,P., Hew,M., Khorasani,N., Torrego,A., Barnes,P.J., Adcock,I., & Chung,K.F. (2008) Relative corticosteroid insensitivity of alveolar macrophages in severe asthma compared with non-severe asthma. *Thorax* **63**, 784-790.

5. Cosio,B.G., Mann,B., Ito,K., Jazrawi,E., Barnes,P.J., Chung,K.F., & Adcock,I.M. (2004) Histone acetylase and deacetylase activity in alveolar macrophages and blood mononocytes in asthma. *Am.J Respir Crit Care Med.* **170**, 141-147.

6. Dean,J.L., Sully,G., Clark,A.R., & Saklatvala,J. (2004) The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation. *Cell Signal.* **16**, 1113-1121.

7. Dobreva ZG, Miteva LD, & Stanilova SA (2009) The inhibition of JNK and p38 MAPKs downregulates IL-10 and differentially affects c-Jun gene expression in human monocytes. *Immunopharmacology and Immunotoxicology* **31**, 195-201.

8. Duan,W., Chan,J.H., McKay,K., Crosby,J.R., Choo,H.H., Leung,B.P., Karras,J.G., & Wong,W.S. (2005) Inhaled p38alpha mitogen-activated protein kinase antisense oligonucleotide attenuates asthma in mice. *Am.J Respir Crit Care Med.* **171**, 571-578.

9. Frantz,B., Klett,T., Pang,M., Parsons,J., Rolando,A., Williams,H., Tocci,M.J., O'Keef,S.J., & O'Neill,E.A. (1998) The Activation State of p38 Mitogen-Activated Protein Kinase Determines the Efficiency of ATP Competition for Pyridinylimidazole Inhibitor Binding. *Biochemistry* **37**, 13846-13853.

10. Galan,A., Garcia-Bermejo,M.L., Troyano,A., Vilaboa,N.E., de Blas,E., Kazanietz,M.G., & Aller,P. (2000) Stimulation of p38 Mitogen-activated
Protein Kinase Is an Early Regulatory Event for the Cadmium-induced Apoptosis in Human Promonocytic Cells. *J.Biol.Chem.* **275**, 11418-11424.

11. Gayo A, Mozoa L, Suáreza A, Tuñón A,L.C., & Gutiérrez C. (1998) Glucocorticoids increase IL-10 expression in multiple sclerosis patients with acute relapse. *Journal of Neuroimmunology* **85**, 122-130.

12. Ge,B., Gram,H., Di Padova,F., Huang,B., New,L., Ulevitch,R.J., Luo,Y., & Han,J. (2002) MAPKK-Independent Activation of p38alpha Mediated by TAB1-Dependent Autophosphorylation of p38alpha. *Science* **295**, 1291-1294.

13. Hew,M., Bhavsar,P., Torrego,A., Meah,S., Khorasani,N., Barnes,P.J., Adcock,I., Fan Chung,K., & for the National Heart Lung and Blood Institute's Severe Asthma Research Program (2006) Relative Corticosteroid Insensitivity of Peripheral Blood Mononuclear Cells in Severe Asthma. *Am.J.Respir.Crit.Care Med.* **174**, 134-141.

14. Irusen,E., Matthews,J.G., Takahashi,A., Barnes,P.J., Chung,K.F., & Adcock,I.M. (2002) p38 Mitogen-activated protein kinase-induced glucocorticoid receptor phosphorylation reduces its activity: role in steroid-insensitive asthma. *J Allergy Clin.Immunol.* **109**, 649-657.

15. John,M., Lim,S., Seybold,J., Jose,P., Robichaud,A., O'Connor,B., Barnes,P.J., & Chung,K.F. (1998) Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. *Am.J Respir Crit Care Med.* **157**, 256-262.

16. Kent,L.M., Smyth,L.J.C., Plumb,J., Clayton,C.L., Fox,S.M., Ray,D.W., Farrow,S.N., & Singh,D. (2009) Inhibition of Lipopolysaccharide-Stimulated Chronic Obstructive Pulmonary Disease Macrophage Inflammatory Gene Expression by Dexamethasone and the p38 Mitogen-Activated Protein Kinase Inhibitor N-cyano-N'-(2-((8-(2,6-difluorophenyl)-4-(4-fluoro-2-methylphenyl)-7-oxo-7,8-dihydropyrido[2,3-d] pyrimidin-2-yl)amino)ethyl)guanidine (SB706504). *J Pharmacol Exp Ther* **328**, 458-468.

17. Koch,A., Giembycz,M., Ito,K., Lim,S., Jazrawi,E., Barnes,P.J., Adcock,I., Erdmann,E., & Chung,K.F. (2004) Mitogen-activated protein kinase modulation of nuclear factor-kappaB-induced granulocyte macrophage-colony-stimulating factor release from human alveolar macrophages. *Am.J Respir Cell Mol.Biol.* **30**, 342-349.

18. Lim,M.Y., Wang,H., Kapoun,A.M., O'connell,M., O'Young,G., Brauer,H.A., Luedtke,G.R., Chakravarty,S., Dugar,S., Schreiner,G.S., Protter,A.A., & Higgins,L.S. (2004) p38 Inhibition attenuates the pro-inflammatory response to C-reactive protein by human peripheral blood mononuclear cells. *J Mol.Cell Cardiol.* **37**, 1111-1114.
19. Lock, S.H., Kay, A.B., & Barnes, N.C. (1996) Double-blind, placebo-controlled study of cyclosporin A as a corticosteroid-sparing agent in corticosteroid-dependent asthma. *Am.J Respir Crit Care Med.* **153**, 509-514.

20. Margutti, S. & Laufer, S.A. (2007) Are MAP kinases drug targets? Yes, but difficult ones. *ChemMedChem* **2**, 1116-1140.

21. Matsuguchi, T., Musikacharoen, T., Ogawa, T., & Yoshikai, Y. (2000) Gene Expressions of Toll-Like Receptor 2, But Not Toll-Like Receptor 4, Is Induced by LPS and Inflammatory Cytokines in Mouse Macrophages. *J Immunol* **165**, 5767-5772.

22. Moore, W.C., Bleecker, E.R., Curran-Everett, D., Erzurum, S.C., Ameredes, B.T., Bacharier, L., Calhoun, W.J., Castro, M., Chung, K.F., Clark, M.P., Dweik, R.A., Fitzpatrick, A.M., Gaston, B., Hew, M., Hussain, I., Jarjour, N.N., Israel, E., Levy, B.D., Murphy, J.R., Peters, S.P., Teague, W.G., Meyers, D.A., Busse, W.W., & Wenzel, S.E. (2007) Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. *J Allergy Clin Immunol.* **119**, 405-413.

23. Nath, P., Leung, S.Y., Williams, A., Noble, A., Chakravarty, S.D., Lueddke, G.R., Medicherla, S., Higgins, L.S., Protter, A., & Chung, K.F. (2006) Importance of p38 mitogen-activated protein kinase pathway in allergic airway remodelling and bronchial hyperresponsiveness. *Eur J Pharmacol.* **544**, 160-167.

24. Saccani, S., Pantano, S., & Natoli, G. (2002) p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat. Immunol.* **3**, 69-75.

25. Shiner, R.J., Nunn, A.J., Chung, K.F., & Geddes, D.M. (1990) Randomised, double-blind, placebo-controlled trial of methotrexate in steroid-dependent asthma. *Lancet.* **336**, 137-140.

26. Sirenko, O.I., Lofquist, A.K., DeMaria, C.T., Morris, J.S., Brewer, G., & Haskell, J.S. (1997) Adhesion-dependent regulation of an A+U-rich element-binding activity associated with AUF1. *Mol.Cell Biol.* **17**, 3898-3906.

27. Sousa, A.R., Lane, S.J., Soh, C., & Lee, T.H. (1999) In vivo resistance to corticosteroids in bronchial asthma is associated with enhanced phosyphorylation of JUN N-terminal kinase and failure of prednisolone to inhibit JUN N-terminal kinase phosphorylation. *J Allergy Clin Immunol* **104**, 565-574.

28. Sweitzer, S.M., Medicherla, S., Almirez, R., Dugar, S., Chakravarty, S., Shumilla, J.A., Yeomans, D.C., & Protter, A.A. (2004) Antinociceptive action of a p38alpha MAPK inhibitor, SD-282, in a diabetic neuropathy model. *Pain.* **109**, 409-419.

29. Szatmary, Z., Garabedian, M.J., & Vilcek, J. (2004) Inhibition of glucocorticoid receptor-mediated transcriptional activation by p38 mitogen-activated protein (MAP) kinase. *J Biol.Chem.* **279**, 43708-43715.
30. Tebo, J., Der, S., Frevel, M., Khabar, K.S., Williams, B.R., & Hamilton, T.A. (2003) Heterogeneity in control of mRNA stability by AU-rich elements. *J Biol. Chem.* **278**, 12085-12093.

31. Tsitoura, D.C. & Rothman, P.B. (2004) Enhancement of MEK/ERK signaling promotes glucocorticoid resistance in CD4+ T cells. *J Clin. Invest* **113**, 619-627.

32. Wang, S.W., Pawlowski, J., Wathen, S.T., Kinney, S.D., Lichenstein, H.S., & Manthey, C.L. (1999) Cytokine mRNA decay is accelerated by an inhibitor of p38-mitogen-activated protein kinase. *Inflamm. Res.* **48**, 533-538.
Figure 1

A. Baseline Cytokine Release (pg/ml)

- Non-Severe Asthma
- Severe Asthma

PBMCs | Alveolar Macrophages

IL-1β | IL-6 | MIP-1α | IL-10 | IL-1β | IL-6 | MIP-1α | IL-10

B. Cytokine (pg/ml)

- LPS
- LPS/Dex

Non-Severe Asthma | Severe Asthma
Figure 2

A. Non-Severe Asthma

B. Severe Asthma
Figure 3

A. Non-severe Asthma

|       | % Cytokine release vs LPS |
|-------|---------------------------|
| IL-1β | *                         |
| IL-6  | *                         |
| MIP-1α| **                        |
| IL-10 | *                         |

B. Severe Asthma

|       | % Cytokine release vs LPS |
|-------|---------------------------|
| IL-1β | *                         |
| IL-6  | **                        |
| MIP-1α| *                         |
| IL-10 | **                        |
Bhavsar et al Figure 4

Phospho p38 (fold increase over NS)

0 2 4 6 8 10

NS  LPS  GW-A  LPS+GW-A  LPS+Dex  LPS+Dex+GW-A

*  **
Bhavsar et al Figure 5

A

GW-A [Log M]

LPS+Dex

% IL-8 Suppression vs LPS

B

Dex [Log M]

LPS+GW-A

% IL-8 Suppression vs LPS

+ Dex $10^{-9}$ M
+ Dex $10^{-8}$ M
+ Dex $10^{-6}$ M
+ GW-A $10^{-10}$ M
+ GW-A $10^{-9}$ M
