PI3K, p38 and JAK/STAT signalling in bronchial tissue from patients with asthma following allergen challenge

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Abstract
Background: Inhaled allergen challenges are often used to evaluate novel asthma treatments in early phase clinical trials. Current novel therapeutic targets in asthma include phosphoinositide 3-kinases (PI3K) delta and gamma, p38 mitogen-activated protein kinase (p38) and Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signalling pathways. The activation of these pathways following allergen exposure in atopic asthma patients is not known.

Methods: We collected bronchial biopsies from 11 atopic asthma patients at baseline and after allergen challenge to investigate biomarkers of PI3K, p38 MAPK and JAK/STAT activation by immunohistochemistry. Cell counts and levels of eosinophil cationic protein and interleukin-5 were also assessed in sputum and bronchoalveolar lavage.

Results: Biopsies collected post-allergen had an increased percentage of epithelial cells expressing phospho-p38 (17.5 vs 25.6%, \(p = 0.04\)), and increased numbers of sub-epithelial cells expressing phospho-STAT5 (122.2 vs 540.6 cells/mm\(^2\), \(p = 0.01\)) and the PI3K marker phospho-ribosomal protein S6 (180.7 vs 777.3 cells/mm\(^2\), \(p = 0.005\)). Type 2 inflammation was increased in the airways post allergen, with elevated levels of eosinophils, interleukin-5 and eosinophil cationic protein.

Conclusions: Future clinical trials of novel kinase inhibitors could use the allergen challenge model in proof of concept studies, while employing these biomarkers to investigate pharmacological inhibition in the lungs.

Keywords: Allergens, Asthma, Bronchoscopy, Immunohistochemistry, JAK-STAT signalling, Kinase, p38 mitogen-activated protein kinase, Phosphoinositide 3-kinase

Background
Novel asthma treatments in early clinical development are often evaluated using the inhaled allergen challenge model [1], which enables rapid evaluation of the potential to suppress allergic inflammation before later phase studies focusing on clinical endpoints. The primary endpoint of allergen challenge studies is usually the fall in lung function caused by allergen inhalation, but biomarkers of lung inflammation can also be measured.

Intracellular kinase inhibition can potentially exert broad immunosuppressive effects on multiple cell types. Examples of kinases that have been considered as therapeutic targets in asthma are phosphoinositide 3-kinases (PI3K) delta and gamma, p38 mitogen-activated protein kinase (p38) and Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT) [2–4]. PI3K\(\delta\) and PI3K\(\gamma\) are predominantly expressed in leukocytes and play a role in lymphocyte differentiation, activation, cell migration and reactive oxygen species production. In animal models of asthma, PI3K\(\delta\) inhibition reduces inflammation and airway remodelling [5]. Furthermore, PI3K\(\delta\) expression is increased in the airways of asthma patients compared to controls [6]. p38
MAPK controls inflammatory gene transcription and translation [2] and is involved in the inflammatory responses of many different cell types to a wide range of stimuli [7–9]. In asthma patients the levels of p38 MAPK activity in the blood have been shown to increase with disease severity [10], while there is also evidence of increased alveolar macrophage and bronchial epithelial p38 MAPK activation in severe asthma [11, 12]. The JAK pathway is activated by pro-inflammatory cytokines leading to phosphorylation of STAT transcription factors [3]. JAK/STAT signalling is essential for lymphocyte differentiation and anti-viral mechanisms [13, 14]. Corticosteroids are currently the most commonly used anti-inflammatory treatment for asthma. However, in vitro studies, using human airway cells, have shown that JAK/STAT-dependent mechanisms are corticosteroid-insensitive [15, 16]. Animal models of allergic asthma show activation of PI3K, p38 MAPK and JAK/STAT pathways in the lungs [17, 18]. However, whether allergen exposure activates these pathways in humans with asthma has not been studied.

This study investigated the activation of PI3K, p38 MAPK and JAK/STAT signalling in lungs of asthma patients after allergen challenge; thus assessing the mechanistic involvement of these pathways during the human allergic response. As allergen challenge studies are often used during early clinical development of novel drugs, we also sought to identify biomarkers of PI3K, p38 MAPK and JAK/STAT activation in the lungs after allergen challenge.

**Methods**

**Patients**

Twelve steroid naïve atopic asthma patients were recruited; nine were male, with mean age of 45.5 and FEV1% predicted of 86.2%. Patient demographics and clinical characteristics can be found in Table 1. All subjects demonstrated a positive skin prick test to either cat or house dust mite extract (Aquagen® SQ, ALK Laboratories, Copenhagen, Denmark); showed methacholine hyperresponsiveness (PC20 < 16 pg/ml); and previously had shown both an early (from 0 to 2 h) and late (from 4 to 10 h) asthma response following allergen challenge, defined as a decrease in FEV1 of >20% and 15% respectively. Allergen challenges were performed using the Cockcroft method [19]. The study was approved by the local research ethics committee (Greater Manchester Central REC reference: 14/NW/0048) and subjects provided written informed consent.

**Study design**

Baseline induced sputum and bronchoscopy samples were collected. Induced sputum samples were processed to obtain supernatant for protein analysis and differential cell count cytospins [20]. Bronchoscopy was performed to collect bronchoalveolar lavage (BAL) fluid for protein analysis and bronchial biopsies for immunohistochemical analysis. Bronchial biopsies were collected from right or left lower lobes. At 6 weeks after the baseline bronchoscopy, an allergen challenge was performed. This time period was to allow for patient recovery following baseline bronchoscopy sample collection. Sputum induction and bronchoscopy were carried out at 4 and 6 h post-allergen challenge respectively; this impeded FEV1 characterisation of the late asthma response.

Immunohistochemistry was used to assess bronchial biopsy expression of the following proteins in both the epithelium and sub-epithelium; phospho-Protein kinase B (pAKT) and phospho-Ribosomal Protein S6 (pRPS6) involved in PI3K signalling; p-p38 and phospho-Heat Shock Protein 27 (p-HSP27) involved in p38 MAPK signalling; and phospho-STAT1s 1, 3, 5 and 6.

Levels of IL-5 and eosinophil cationic protein (ECP) were assessed in sputum supernatants and bronchoalveolar lavage by MesoScaleDiscovery assay at SGS Cepheurope, Saint Benoit Cedex, France. Sputum differential cell counts were assessed by Rapi-Diff stain [20].

**Cockcroft allergen challenge**

Allergen (Aquagen® SQ, ALK Laboratories, Copenhagen, Denmark) inhalations using the tidal breathing method were performed with a deVilbiss nebuliser operated by air and at a flow rate to give an output of 0.13 ml/min. The subjects wore a nose-clip and aerosol was inhaled through a mouth-piece during tidal volume breathing (VT). The starting concentration for the allergen was determined with results of the skin sensitivity test and the methacholine PC20 according to the formula published by Cockcroft et al., 1987. The starting concentration was 2 doubling doses less than predicted to cause a 20% fall in FEV1 during the EAR. FEV1 was measured twice, one minute apart, 10 min after the allergen was administered. If the FEV1 fell below...
by < 15%, the next dose of allergen was administered. If
the FEV1 fall was ≥ 15% and < 20%, FEV1 measurements
were performed at 15 mins post allergen to see if a ≥ 20% fall was achieved. When the FEV1 fell by ≥ 20%
or the highest dose of allergen was administered, the
allergen challenge was discontinued and serial FEV1
measurements were performed for 4 h before performing
a sputum induction. An early asthmatic response (EAR –
FEV1 fall ≥ 20%) was assessed after 2 h.

**Methacholine challenge**

Methacholine challenges, using Methacholine chloride,
Stockport Pharmaceuticals, Stockport, UK, were performed
if the FEV1 was ≥ 65% predicted. Subjects inhaled doubling
concentrations of methacholine (0.03125 to 16 mg/ml) for
2 min at V̇E with a nose clip, at 5 min intervals using the
deVilbiss nebuliser until there was a fall in FEV1 of ≥ 20%.
FEV1 was measured at 30 and 90 s intervals after each
allergen administration and the highest value used for cal-
culated FEV1 fall. Initially, a diluent control was used. Sub-
jects with a post-diluent decrease ≥ 10% did not continue
to the methacholine challenge. The provocation concentra-
tion of methacholine required to cause a fall in FEV1 of
20% (PC20) was calculated from the dose response curve.

**Serial skin prick test**

The skin sensitivity test was performed to determine the
allergen PC20 estimation for the tidal breathing chal-
lenge. Duplicate skin prick tests were performed with
the fold dilutions of the same allergen (Aquagen® SQ,
ALK Laboratories, Copenhagen, Denmark) used for in-
halation. The diameter of each wheal diameter was cal-
culated from the average of the two. Cutaneous skin
sensitivity was defined as the concentration producing a
mean wheal diameter of 2 mm and was obtained from a
plot of the log concentrations versus wheal size.

**Sputum induction**

Sputum samples were obtained at the start of the study
(visit 1) and at 4 h post allergen challenge at visit 3. Spu-
tum induction was performed after administration of
200-400 μg of Salbutamol. Increasing saline concentra-
tions (3%, 4%, 5%) was given to the patient as 3 nebulisa-
tions (Flaem Nuova EasyNeb II, Milan, Italy) each
lasting for 5 min. Spirometry (Micro-loop®, Carefusion,
Basingstoke, UK) was performed prior to sputum induc-
tion and after each nebulisation cycle as a safety precau-
tion to assess the effect of inhaled saline on FEV1.

**Sputum processing**

Selected sputum was weighed, and samples greater than
0.05 g was mixed with eight volumes of phosphate buffered
saline (PBS) before centrifugation. Supernatants were col-
lected and stored at -80 °C until required for protein
analysis. Remaining cells were mixed with 0.1% DTT for
15 min. Harvested cells were re-suspended in cold PBS so
that a cell count could be performed using trypan blue to
assess the number of viable cells. Cytospin slides were pre-
pared for differential count. Cytospin preparations were air
dried, fixed with methanol and stained with Rapi-diff
(Triangle, Skelmersdale, UK). Each reader scored 400 cells.
This was used to determine the percentage of squamous
cells as a measure of sputum quality. Samples with < 50%
squamous cells were scored as acceptable. The results were
expressed as a percentage of the total non-squamous count,
and a total cell count/g of sputum.

**Bronchoscopy and sample processing**

Bronchoscopy was performed after the subjects had been
sedated. BAL was collected from the right or left upper
lobe. The bronchoscope was wedged in the bronchus
and a maximum of 4 × 60 ml aliquots of pre-warmed
sterile 0.9% saline solution were instilled. The aspirated
fluid was stored on ice before filtration (100 µm filter,
Becton Dickenson, Oxford, UK). The filtrate was centri-
fuged (400 g/10 min at 4 °C) and the BAL fluid was
stored at -80 °C in 1 ml aliquots for biomarker analysis.
The cell pellet resuspended in PBS and viable cell counts
were determined by trypan blue exclusion (Neubauer
hemocytometer), and the suspension adjusted to 0.5 × 10⁶
BAL cells/ml. Cytospins for differential cell counts were
made using 100µl of cell suspension. Bronchial biopsies
were collected from the right or left lower lobes.

**Immunohistochemistry**

Bronchial biopsies were fixed overnight in 10% neutral buff-
ered formalin (CellPath, Powys, UK), and embedded in par-
affin wax (Surgipath, Milton Keynes, UK). 3 µm tissue
sections were cut and lifted onto a polysine coated glass
slides, before dewaxing in xylene and rehydrated with de-
creasing concentrations of alcohol. Following heat induced
epitope retrieval (20 min in microwave at 800 W); tissues
were stained overnight with primary antibody, before detec-
tion with biotinylated anti-rabbit IgG secondary antibody
(Vector Laboratories, Peterborough, UK). Staining was visu-
alised using avidin biotin peroxidase complex (Vector
Laboratories). All sections were counterstained with Gill’s II
haematoxylin (Leica, Milton Keynes, UK). Quantification
was performed using the ImagePro Plus 6.0 software.
All antibodies were purchased from Cell Signalling
Technology, Davers, USA, except for pSTAT6 (Life
Technology, Paisley, UK): pAKT product #9271; pRPS6
product #5364; p-p38 product #9211; p-HSP27 product
#2401; phospho-STAT 1, 3, 5 and 6 product codes 9167,
9145, 9359 and 700,247, respectively. Sub-epithelial cell
counts for neutrophils (neutrophil elastase), eosinophils
(Luna stain), CD4⁺ and CD8⁺ cells were performed as
previously described [21]. Positive staining was confirmed
Fig. 1 Inflammatory cell counts in sub-epithelial bronchial tissue at baseline and post-allergen (n = 11). Bronchial biopsies were collected from 11 asthma patients at baseline and 6 h post allergen challenge, with CD4, CD8 and neutrophil numbers being assessed by immunohistochemistry and eosinophils with Luna staining. Data presented as mean (sd). Comparisons between Baseline and Post-allergen by paired T-test. Images of staining are at × 200 magnification; Black bars represent 100 μm. Magnified images and red arrows illustrate positive staining. Negative control images represent isotype controls results for CD4, CD8 and neutrophil elastase, and non-Luna staining for eosinophils.

| Cell Type | CD4 | CD8 | Eosinophil | Neutrophil |
|-----------|-----|-----|------------|------------|
| Baseline  | 78.6 (36.0) | 117.8 (83.6) | 4.1 (8.2) | 1.7 (3.3) |
| Post-Allergen | 95.8 (54.7) | 130.5 (102.5) | 5.1 (4.7) | 1.5 (2.2) |
| p         | 0.68 | 0.69 | 0.73 | 0.85 |

Fig. 2 Levels of PI3K, p38 and JAK/STAT markers in bronchial epithelium at baseline and post allergen (n = 11): Bronchial biopsies were collected from 11 asthma patients at baseline and 6 h post allergen challenge, with protein expression analysed in the epithelium (a) by immunohistochemistry. Data presented as mean values with standard deviation. Individual patient’s data for the statistically significant changes in pp38 are shown in (b). Comparisons between baseline and post allergen were by paired T-tests or Wilcoxon tests (#), as appropriate *p < 0.05.
by use of two negative controls: (i) exclusion of primary antibody and (ii) use of isotype control antibody.

**Differential cell count**
Sputum and BAL cell cytospins were fixed in methanol for 10 min and left to air dry, before being stained with Rapi-Diff (GCC Diagnostics) using the following method: (1) stain slide for 2 min in solution A; (2) wash slide in distilled water; (3) stain slide for 2 min in solution B; (4) wash slide under tap water. Slides are then mounted using DPX and cover slipped. 400 non-squamous cells were counted per slide. Neutrophils, macrophages, eosinophils, lymphocytes and bronchial epithelial cells differentiated based on morphology and staining.

**Statistical analysis**
Data distribution was assessed by Kolmogorov-Smirnov test. Data with non-parametric distribution are highlighted in figures with an asterisk. Changes in cell counts and protein levels pre- and post-allergen challenge were analysed by two-sided paired T-tests or Wilcoxon tests, where appropriate. All analysis was carried out using Prism 7.0 software (Graphpad, La Jolla, California, USA). A *p*-value of less than 0.05 was deemed significant.

**Results**
One subject was withdrawn after not tolerating the baseline bronchoscopy, with 11 completing the study. All subjects demonstrated an early asthma response with a fall in FEV₁ of > 20% within 20 min of allergen inhalation, with a mean maximum fall of 29.2%.

Paired baseline and post-allergen sputum samples were successfully collected from 10 of the 11 patients. Allergen challenge increased sputum eosinophil percentage (from mean 1.1% to 3.8%; *p* = 0.019) and cell count (from median 0.01 to 0.12 × 10⁶ cells/g sputum;
Neutrophil percentage and cell counts also increased (from mean 37.9 to 63.4%, \( p = 0.008 \), and from 0.8 to \( 2.0 \times 10^6 \) median neutrophils/g sputum: \( p = 0.05 \)). The macrophage percentage decreased (from mean 49.9 to 28.6%, \( p = 0.02 \)) after allergen challenge. There were no changes in sputum lymphocyte or epithelial cell numbers.

Type 2 inflammation was increased by allergen challenge; sputum supernatant ECP (57 vs 169 ng/ml; \( p = 0.049 \)) and IL-5 (0.9 vs 19.9 pg/ml; \( p = 0.016 \)) levels were higher after allergen challenge. BAL ECP levels were increased by allergen challenge (1.9 vs 3.2 ng/ml; \( p = 0.014 \)). The higher levels measured in sputum supernatant was probably due to the know dilution effect of BAL, but the median fold change in ECP was similar in sputum supernatant and BAL (2.7-fold and 2.0-fold, respectively). Levels of IL-5 in BAL were undetectable, both at baseline and post allergen. Allergen exposure had no effect on neutrophil, macrophage, eosinophil and lymphocyte cell numbers in bronchial tissue (Fig. 1).

In bronchial epithelium there was a significant increase in the percentage of cells expressing p-p38 post-allergen (\( p = 0.04 \); Fig. 2). The percentage of pRPS6 positive epithelial cells was also increased, but this did not reach significance (\( p = 0.06 \)). Numbers of sub-epithelial cells expressing pRPS6 and pSTAT5 were increased post-allergen (\( p = 0.01 \) and \( p = 0.005 \) respectively, Fig. 3). Numbers of pAKT, p-p38 and pSTAT6 positive sub-epithelial cells were also increased, but these did not reach significance (\( p = 0.06, p = 0.07 \) and \( p = 0.06 \) respectively). Other proteins analysed in epithelium (pHSP27, pAKT, pSTATs 1, 3, 5 and 6) or sub-epithelium (pHSP27, pSTATs 1 and 3) by immunohistochemistry did not show any change after allergen challenge. Representative images of bronchial tissue staining are shown in Fig. 4.

**Discussion**

There was an increase in p-p38 MAPK expression in the bronchial epithelia after allergen challenge, while in the sub-epithelium there was evidence of PI3K activation (pRPS6) and JAK/STAT activation (pSTAT5). These observations support involvement of these kinase pathways in the allergic response, but with different roles in the epithelium compared to the sub-epithelium.

Phospho-p38 MAPK expression is increased in the bronchial epithelium of patients with severe asthma [12, 22]. We now show p38 MAPK activation at the same anatomical location after allergen challenge. Overall, these findings suggest that epithelial p38 MAPK activation contributes to the inflammatory cascade in asthma caused by environmental triggers, such as allergen exposure. In vitro studies using human bronchial epithelial cells have shown that the house dust mite antigen, DerP1, induces p38-dependent cell apoptosis and intracellular oxidative stress [5], while in mice p38 is essential for allergen induced epithelial production of IL-25 and thymic stromal lymphopoietin (TSLP), which are both initiators of the Type-2 allergic response in asthma [10].
There is evidence that PI3K activation is increased in severe asthma [23]. PI3Kδ has a central role in T-cell activation [4]. The allergic response involves T-helper 2 cell activation, suggesting that PI3K activation could occur after allergen challenge. RPS6 is a terminal signaling protein of the PI3K/AKT/mTOR pathway, involved in protein synthesis and cell cycle progression [24]. Increased pRPS6 levels in the sub-epithelium suggest PI3K activation by allergen challenge, perhaps due to increased T-cell activity [25], although we did not specifically evaluate this.

Increased pSTAT5 expression suggests JAK/STAT pathway involvement in sub-epithelial leukocyte activation after allergen challenge. In murine lungs, STAT5 in dendritic cells is essential for TS-LP-induced Th2 responses following allergen exposure [26], while STAT5 is also involved in mast cell functions, such as expression of the high affinity IgE receptor [27]. Other STAT proteins did not display changes that reached statistical significance.

The increased sub-epithelial expression of pSTAT5 and pRPS6 after allergen challenge was not due to an increase in cell numbers, as there were no increases in sub-epithelial leukocyte numbers. There are inconsistent reports of changes in leukocyte numbers in the bronchial mucosa after allergen challenge, probably due in part to differences in the time-point of sampling [28, 29]. This contrasts with the increase in luminal eosinophilic inflammation after allergen challenge, which we and others have observed [30]. Although we used a limited sample size, it was sufficiently large to observe the expected upregulation of airway luminal eosinophilic inflammation, evidenced by increased eosinophil numbers as well as IL-5 and ECP levels [31].

The expression of protein phosphorylation can be very transient e.g. pAKT expression can be short-lived in contrast to pRPS6 expression [32]. This may explain why we did not observe positive results for all the biomarkers within a kinase pathway e.g. pRPS6 expression, but not pAKT, in the sub-epithelium. We were limited in the quantity of tissue available to perform validation experiments using a different technique, such as Western blotting. Nevertheless, our results demonstrate biomarkers within these kinase pathways that show the most promise for application during the study of allergic responses in human lungs.

Conclusions
This study presents candidate biomarkers of kinase activation during the asthmatic allergic response. Future clinical trials of novel kinase inhibitors could consider using the allergen challenge model in proof of concept studies, while employing these biomarkers to understand the pharmacological effects of kinase inhibition in the lungs.
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