

# Remodeling and Airway Hyperresponsiveness but Not Cellular Inflammation Persist after Allergen Challenge in Asthma

Harsha H. Kariyawasam<sup>1,2,3</sup>, Maxine Aizen<sup>1,3</sup>, Julia Barkans<sup>1,3</sup>, Douglas S. Robinson<sup>1,2,3</sup>, and A. Barry Kay<sup>2,3</sup>

<sup>1</sup>Allergy and Clinical Immunology Section, <sup>2</sup>Leukocyte Biology Section, and <sup>3</sup>Medical Research Council and Asthma U.K. Centre in Allergic Mechanisms of Asthma, Faculty of Medicine, National Heart and Lung Institute, Imperial College London, South Kensington, London, United Kingdom

**Rationale:** Airway hyperresponsiveness (AHR) increases up to 2 weeks after allergen inhalational challenge of subjects with asthma who show a late-phase asthmatic reaction (dual responders). Cellular inflammation and airway remodeling are increased 24 hours after allergen challenge.

**Objectives:** To determine whether persistence of increased AHR is associated with persistent activation of remodeling and enhanced inflammation.

**Methods:** Fiberoptic bronchoscopy was performed at baseline and at 24 hours and 7 days after allergen inhalational challenge of dual responders with mild-moderate asthma. At each time point, AHR, spirometry, and expression of tenascin (extracellular matrix protein), procollagen I, procollagen III, and heat shock protein (HSP)-47 (markers of collagen synthesis), and  $\alpha$ -smooth muscle actin (myofibroblasts) were evaluated as markers of activation of airway remodeling, together with numbers of mucosal major basic protein-positive eosinophils, CD68<sup>+</sup> macrophages, CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T cells, elastase-positive neutrophils, and tryptase-positive mast cells. **Measurements and Main Results:** AHR was increased from baseline at 24 hours and 7 days after allergen challenge. Reticular basement membrane tenascin expression was elevated at 24 hours and returned to baseline levels at 7 days. Reticular basement membrane procollagen III expression was significantly elevated at 7 days. Expression of procollagen I, HSP-47, and  $\alpha$ -smooth muscle actin were all higher at 7 days compared with 24 hours. At 24 hours, eosinophil, macrophage, neutrophil, and CD3<sup>+</sup> T cells were increased but had returned to baseline by 7 days.

**Conclusions:** In dual responders with asthma, the 24-hour increase in airway wall cellular inflammation after allergen challenge resolves by 7 days, whereas the increases in AHR and markers of remodeling persist.

**Keywords:** asthma; airway hyperresponsiveness; inflammation

Airway hyperresponsiveness (AHR) is an exaggerated narrowing of the airway in response to specific and nonspecific stimuli. It is a ubiquitous feature of asthma and can serve as an indicator of asthma severity. In general, the diurnal variability of airflow obstruction and symptoms of chest tightness, cough, and wheeze on exposure to irritants such as cold air, smoke, and perfumes are a result of AHR. Both airway inflammation and airway remodeling are implicated in the pathogenesis of AHR but the exact relationship and contribution to AHR remain undetermined.

(Received in original form September 4, 2006; accepted in final form February 1, 2007)

Supported by the Imperial College Trust Fund. D.S.R. is partly funded by a Research Leave Award from the Wellcome Trust UK.

Correspondence and requests for reprints should be addressed to A. Barry Kay, Emeritus Professor of Allergy and Clinical Immunology, Leukocyte Biology Section, Sir Alexander Fleming Building, Imperial College London, London, SW7 2AZ, UK. E-mail: a.b.kay@imperial.ac.uk

Am J Respir Crit Care Med Vol 175, pp 896–904, 2007

Originally Published in Press as DOI: 10.1164/rccm.200609-1260OC on February 1, 2007  
Internet address: www.atsjournals.org

## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

The relationship of airway hyperresponsiveness (AHR) to the activation and resolution of inflammatory and remodeling events in asthma remains undefined. It is suggested that AHR can be dissociated from cellular inflammation but not remodeling.

### What This Study Adds to the Field

Increases in AHR after allergen challenge remain associated with remodeling but not cellular inflammation. Persistence of AHR is not dependent on sustained inflammatory cell recruitment.

Allergen challenge in sensitized patients with asthma leads to immediate airway bronchoconstriction termed the “early asthmatic response” (EAR), which is maximal within 30 minutes and is resolved within 1 to 3 hours. Up to 50% of these individuals will experience a second delayed phase of bronchoconstriction, leading to the dual asthmatic reaction (DAR). The DAR has been defined as a fall in the FEV<sub>1</sub> of 15% from the baseline value between 3 and 7 hours (1). The DAR is associated with increases in AHR, which are not seen in those individuals with isolated EAR (2). Allergen inhalation challenge induces infiltration of airway mucosal inflammatory cells as shown by increases in eosinophils, T cells, and neutrophils in bronchoalveolar lavage fluid (3, 4) and the lamina propria of the airway wall (5). Some studies have indicated that airway inflammation is more pronounced in patients with DAR than in those with isolated EAR, indicating that infiltrating leukocytes, especially eosinophils, may be related to the increased AHR (6, 7), modeling disease exacerbations. We have recently demonstrated that activation of airway remodeling pathways is an acute event and is up-regulated at the same time as inflammation in dual responders with asthma 24 hours after allergen inhalational challenge (8). What is not currently known is whether the sustained increases in AHR after allergen challenge are associated with the persistence of increased airway remodeling, particularly in the presence or absence of inflammation. For these reasons, we have obtained bronchial biopsies at baseline and 24 hours and 7 days after allergen challenge in a group of DAR volunteers to evaluate whether sustained increases in AHR after allergen challenge are associated with the persistence of airway inflammation and remodeling. Some of the results of these studies have been previously reported in the form of an abstract (9). Our data show that persistent recruitment of infiltrating inflammatory cells is not essential to the maintenance of allergen-induced increases

of AHR and that airway remodeling can remain associated with AHR at a time point when there is resolution of cellular inflammation to baseline levels.

## METHODS

### Volunteers

The study was approved by the Royal Brompton and Harefield Hospital Ethics Committee, and volunteers gave written, informed consent.

Thirteen atopic patients with asthma with a history of atopic asthma together with either a 15% increase in FEV<sub>1</sub> to  $\beta_2$ -agonist or methacholine PC<sub>20</sub> (the provocative concentration causing a 20% fall in FEV<sub>1</sub>) of 8 mg/ml or less were recruited: data are presented for the nine subjects who developed a late asthmatic response to allergen challenge. The baseline FEV<sub>1</sub> was 70% or greater of predicted and all subjects demonstrated positive skin-prick tests (SPTs; wheal size  $\geq$  3 mm) to one or more of the aeroallergens house dust mite, cat dander, or grass (ALK, Hørsholm, Denmark). Volunteers sensitive to pollens were studied outside of the season. Volunteers were controlled with only rescue  $\beta_2$ -agonists at the time of study and had no clinical features of infection for at least 4 weeks before the baseline bronchoscopy and none throughout the study. All were nonsmokers.

### Study Design

The study outline is summarized in Figure 1. Methacholine challenge was performed according to the standards set by the American Thoracic Society guidelines (10). Allergen challenges were performed with either mixed grass, *Dermatophagoides pteronyssinus* (Allergopharma, Reinbek, Germany), or cat dander (Leti, Madrid, Spain) extracts chosen based on history, SPT, and allergen-specific serum IgE. An incremental dosing schedule as previously described was used (11).

A decrease in the FEV<sub>1</sub> of more than 15% of the postsaline reference was defined as an EAR. An LAR was defined as a fall in the FEV<sub>1</sub> of 15% or more from the postsaline reference value at a single time point between 3 and 7 hours after allergen inhalation (1).

Fiberoptic bronchoscopy with bronchial biopsies was performed as previously described (8) at baseline, 24 hours after allergen challenge, and 7 days after allergen challenge. All bronchoscopies were conducted at 8.30 A.M., and the order of sampling was randomized.

### Immunohistochemistry

Tissue processing and immunostaining were performed as previously described (3, 12). Monoclonal antibodies used were anti-CD3, CD4, CD8, CD68, neutrophil elastase, human mast cell tryptase (all from Dako, High Wycombe, UK), and eosinophil major basic protein (MBP) (BMK-13; in-house) with isotype controls. Remodeling markers used were heat shock protein (HSP)-47 (clone M16.10A1; Stressgen, Victoria, BC, Canada), procollagen I (Chemicon, Harrow, UK), and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Dako). Reticular basement membrane (RBM) tenascin (Monosan, Uden, The Netherlands) and procollagen III (Chemicon) deposition was evaluated using a Leica TCS SP confocal microscope (Leica, Heidelberg, Germany) and a Scion Image Analysis software package (Scion Corporation, Frederick, MD) as previously published (8, 13). Cell counts (cells per millimeter squared) were determined by counting the whole section in a blinded fashion by a single observer using an Olympus BH-2 microscope (Olympus, New York, NY).

### Statistical Analysis

The methacholine PC<sub>20</sub> was expressed as the geometric mean (range). Cell counts and RBM measurements are expressed as the median (interquartile range). All paired within-subject data were analyzed using the Wilcoxon signed rank test. The data were also analyzed using a mixed model to assess the change over time. In this model, patients were entered as a random effect, with time as a fixed effect. Correlation coefficients were obtained using Spearman's rank order method. Correlations were performed between AHR at 24 hours and AHR at 7 days against cellular counts and remodeling markers. A GraphPad Prism software package was used (GraphPad Software Ver. 4, Inc., San Diego, CA). Significance was accepted as  $p < 0.05$ .

## RESULTS

### Increased AHR Is Sustained Up to 7 Days

Volunteer characteristics are summarized in Table 1. There were five males and four females with a median age of 24 (range, 19–46) years and median FEV<sub>1</sub>% predicted of 97% (range, 88.70–118.2%). One volunteer did not complete the final bronchoscopy and the paired-data analysis has taken this into account.

The airway responses to allergen are summarized in Figure 2. The median decrease in FEV<sub>1</sub> measured from baseline was  $-16.1\%$  (range,  $-21.7$  to  $-2$ ) at 24 hours ( $p = 0.02$ ), and  $-5.04\%$  ( $-8$  to  $+7.8$ ) at 7 days ( $p =$  not significant) (Figure 2A). AHR was increased at both 24 hours and 7 days compared with baseline after allergen challenge (Figure 2B). The methacholine PC<sub>20</sub> (geometric mean  $\pm$  range) decreased from 1.707 (0.71–4.00) mg/ml at baseline to 0.3828 (0.0625–2.00) mg/ml at 24 hours ( $p = 0.004$ ) and 0.4423 (0.20–1.260) mg/ml at 7 days ( $p = 0.02$ ).

### Airway Remodeling Remains Associated with AHR

The change in expression of markers of airway remodeling is presented in Table 2 and Figure 3. The expression of RBM tenascin increased at 24 hours after allergen challenge ( $p = 0.004$ ) but approached baseline levels again by Day 7 (Figure 3A). RBM procollagen III expression was significantly increased at 7 days ( $p = 0.01$ ) (Figure 3B). There was an upward trend in procollagen I expression at 24 hours postallergen, whereas the increases in HSP-47 expression in fibroblasts were significant ( $p = 0.04$ ). Further significant increases in procollagen I and HSP-47 expression were observed at Day 7 ( $p = 0.0078$  and  $p = 0.0078$ , respectively, compared with baseline; Figures 3C and 3D). There was an upward trend in the number of fibroblasts expressing  $\alpha$ -SMA 24 hours after allergen challenge ( $p = 0.09$ ) that was sustained at the 7-day time point ( $p = 0.1$ ). Fibroblasts were identified morphologically as being fusiform in shape, with elongated nuclei, as previously published (8).

Figure 4 shows an example of confocal micrographs of tenascin and procollagen III expression in the RBM at baseline (Figures 4A and 4D, respectively), at 24 hours after allergen challenge (Figures 4B and 4E, respectively) and at 7 days

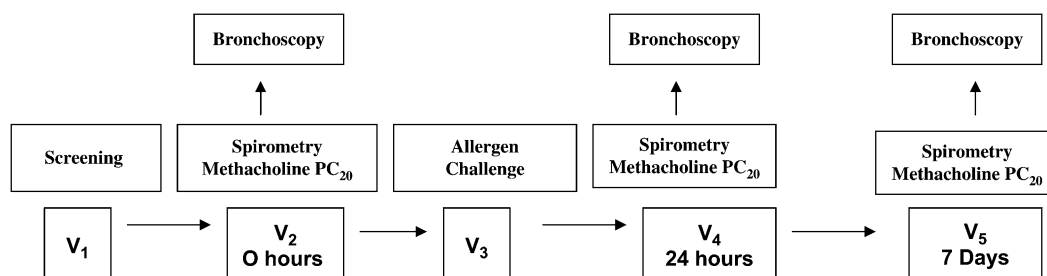


**Figure 1.** Summary of the study design. After a screening visit, subjects with asthma underwent a baseline bronchoscopy at Visit 2 (V<sub>2</sub> 0 hours). Visit 4 (V<sub>4</sub>) and Visit 7 (V<sub>7</sub>) were 24 hours and 7 days after allergen challenge, respectively. Spirometry was performed and methacholine-induced AHR was measured before each bronchoscopy.

TABLE 1. VOLUNTEER DEMOGRAPHY AND AIRWAY RESPONSES TO ALLERGEN CHALLENGE

| Volunteer | Age | Sex | FEV <sub>1</sub> % Predicted | PC <sub>20</sub> Methacholine (mg/ml) | Allergen-specific IgE (IU/ml) | Allergen Dose (BU) | Maximum FEV <sub>1</sub> % Decrease |     |
|-----------|-----|-----|------------------------------|---------------------------------------|-------------------------------|--------------------|-------------------------------------|-----|
|           |     |     |                              |                                       |                               |                    | EAR                                 | LAR |
| 1         | 23  | F   | 95.7                         | 6.16                                  | 36.4 (H)                      | 400                | -30                                 | -42 |
| 2         | 31  | M   | 101.9                        | 0.88                                  | 100.2 (H)                     | 175                | -19                                 | -36 |
| 3         | 46  | M   | 118.2                        | 8                                     | 11.6 (G)                      | 162                | -27                                 | -25 |
| 4         | 21  | F   | 88.7                         | 1.74                                  | 20.5 (H)                      | 1325               | -41                                 | -39 |
| 5         | 44  | M   | 101.2                        | 2.89                                  | 3.63 (C)                      | 412                | -36                                 | -36 |
| 6         | 27  | F   | 97                           | 1.08                                  | 62.10 (H)                     | 1325               | -39                                 | -32 |
| 7         | 19  | F   | 90.3                         | 1.4                                   | 0.93 (C)                      | 5325               | -56                                 | -16 |
| 8         | 29  | M   | 92.9                         | 0.61                                  | 8.53 (H)                      | 2125               | -46                                 | -35 |
| 9         | 22  | M   | 95.13                        | 3.2                                   | 54.80 (C)                     | 390                | -23                                 | -23 |

Definition of abbreviations: BU = biologic units; C = cat dander; EAR = early asthmatic response; F = female; G = mixed grass pollen; H = house dust mite; M = male.

(Figures 4C and 4F, respectively). Figure 5 demonstrate fibroblast-like cells in a selected volunteer expressing HSP-47 at baseline, 24 hours, and 7 days (Figures 5A–5D), and procollagen I and  $\alpha$ -SMA in fibroblast-like cells at Day 7 (Figures 5E and 5F, respectively).

There was a significant correlation between AHR and HSP-47 expression in fibroblasts at the 24-hour time point ( $r = -0.8$ ,  $p = 0.02$ ) only. There was a nonsignificant trend between increased AHR and procollagen I cellular expression at the 7day time point ( $r = -0.4$ ,  $p = 0.3$ ). AHR did not correlate with either cellular inflammation or RBM tenascin and procollagen III expression.

#### Airway Inflammation Returns to Baseline at 7 Days

The cellular median counts (interquartile range) in bronchial biopsies are summarized in Table 3. Significant increases in MBP-positive eosinophils ( $p = 0.02$ ), CD68<sup>+</sup> macrophages ( $p = 0.01$ ), and CD3<sup>+</sup> T cells ( $p = 0.004$ ) from baseline at

24 hours were observed after allergen challenge (Figures 3E, 3F, and 3G, respectively). There were no significant increases at 7 days with cellular infiltration returning to baseline levels. In addition, there was a small but significant increase in neutrophils at 24 hours ( $p = 0.03$ ) (Figure 3H) but no significant increase in either CD4<sup>+</sup> or CD8<sup>+</sup> T cells at either 24 hours or 7 days after allergen challenge. Mast cells were counted in both the whole section and in association with airway smooth muscle (ASM). The presence of smooth muscle in the biopsy was confirmed by staining for SMA. No significant increases in mast cell numbers were observed in either the total mast cell or smooth muscle at either 24 hours or 7 days after allergen challenge (Table 3).

Analysis of data using a mixed modeling approach was used to assess whether the change per day for each of the above variables was significant. The observed changes in each of the parameters per day, based on the measurements at baseline, 24 hours, and 7 days, are summarized in Table 4. The changes per day for AHR, procollagen I, procollagen III, and HSP-47 were significant.

## DISCUSSION

In this study, we show that, whereas the allergen-induced increases in airway inflammation in dual responders with asthma

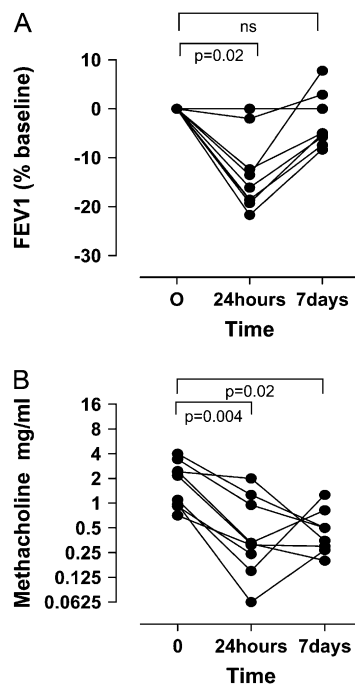


Figure 2. The changes from baseline in FEV<sub>1</sub> (A) and methacholine PC<sub>20</sub> (B) 24 hours and 7 days after allergen challenge.

TABLE 2. SUMMARY OF AIRWAY REMODELLING MARKER EXPRESSION

| Remodeling Marker                                      | 0 Hours                | 24 Hours                | 7 Days                 |
|--------------------------------------------------------|------------------------|-------------------------|------------------------|
| Tenascin, RBM units                                    | 854.2<br>(386.5–1,055) | 1,368*<br>(1,023–1,989) | 942<br>(571.6–1,343)   |
| Procollagen III, RBM units                             | 2,051<br>(1,374–2,657) | 2,483<br>(1,550–2,965)  | 2465*<br>(1,845–3,570) |
| Procollagen I <sup>+</sup> , all cells/mm <sup>2</sup> | 88.0<br>(13.3–124.4)   | 125.7<br>(57.2–266.5)   | 232*<br>(64–332)       |
| HSP-47 <sup>+</sup> , all cells/mm <sup>2</sup>        | 48<br>(33–118)         | 121.6<br>(79.43–173.8)  | 202.7*<br>(123.6–520)  |
| HSP-47 <sup>+</sup> fibroblasts/mm <sup>2</sup>        | 2.67<br>(1–12)         | 16*<br>(4.4–34.7)       | 83.56*<br>(36–452)     |
| $\alpha$ -SMA <sup>+</sup> fibroblasts/mm <sup>2</sup> | 13.3<br>(4–49.15)      | 37.7<br>(28.70–59.20)   | 47.95<br>(13.5–97.35)  |

Definition of abbreviations:  $\alpha$ -SMA =  $\alpha$ -smooth muscle actin; HSP = heat shock protein; RBM = reticular basement membrane.

The cell counts (cells/mm<sup>2</sup>) and RBM units (thickness  $\times$  density) are expressed as the median (interquartile range).

\*  $p < 0.05$  compared with the cell count at baseline.

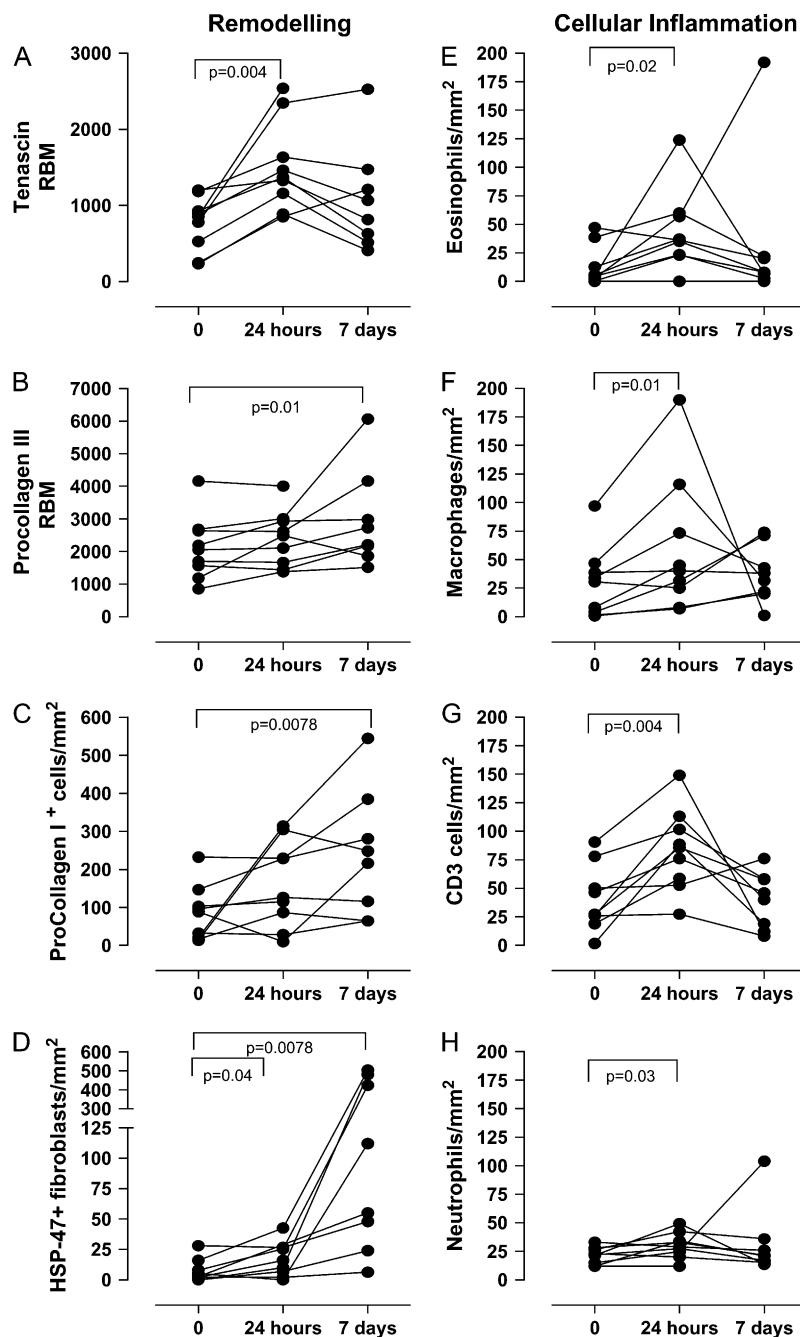


**Figure 3.** Markers of airway remodeling and inflammatory cells 24 hours and 7 days after allergen challenge. Reticular basement membrane (RBM) tenascin and procollagen III (measured as density  $\times$  thickness of subbasement membrane staining by image analysis): procollagen I-positive (ProCollagen I<sup>+</sup>) cells and the collagen chaperone heat shock protein (HSP)-47<sup>+</sup> fibroblasts (as positive stained cells per mm<sup>2</sup>) are shown in A through D, respectively. Fibroblasts were fusiform with elongated nuclei. The numbers of major basic protein-positive eosinophils, CD68<sup>+</sup> macrophages, CD3<sup>+</sup> T cells, and elastase-positive neutrophils in bronchial biopsies from volunteers at baseline, 24 hours, and 7 days after allergen challenge are also shown (E through H, respectively).

were virtually resolved by 7 days, increases in AHR and the expression of collagen markers of airway remodeling persisted. We therefore demonstrate, for the first time, that AHR can be dissociated from cellular inflammation while remaining associated with sustained airway remodeling.

We confirmed the original observation of Cockcroft and colleagues (2) that allergen inhalation increases in AHR in dual responders with asthma are sustained for at least 7 days. The temporal association between increased inflammatory cell infiltration and increased AHR at 24 hours after allergen challenge in dual responders (1, 6, 14) has led to the suggestion of a causal relationship between the two. However, our current findings suggest that increases in airway wall cellular inflammation do not need to be sustained for persistence of increased AHR. Other less direct evidence supports the view that cellular inflammation,

particularly eosinophil infiltration (15), does not necessarily directly relate to AHR. For example, there has been a failure to demonstrate a convincing correlation between the two variables, with several studies having found no convincing association (15–19). In both allergic rhinitis and eosinophilic bronchitis, there is airway inflammation that is indistinguishable from asthma, but these patients do not develop AHR (20, 21). It is possible that the activation status of eosinophils may differ between asthma and other diseases. Several mouse models of allergen-induced airway inflammation demonstrate dissociation of AHR from inflammatory cells. In a chronic airway challenge model, eosinophilic inflammation persisted despite a fall in AHR to baseline values, whereas AHR was unaffected by ablation of eosinophils in GATA-1 knockout animals (22, 23). In a similar eosinophil-deficient model, AHR could be induced independent of airway

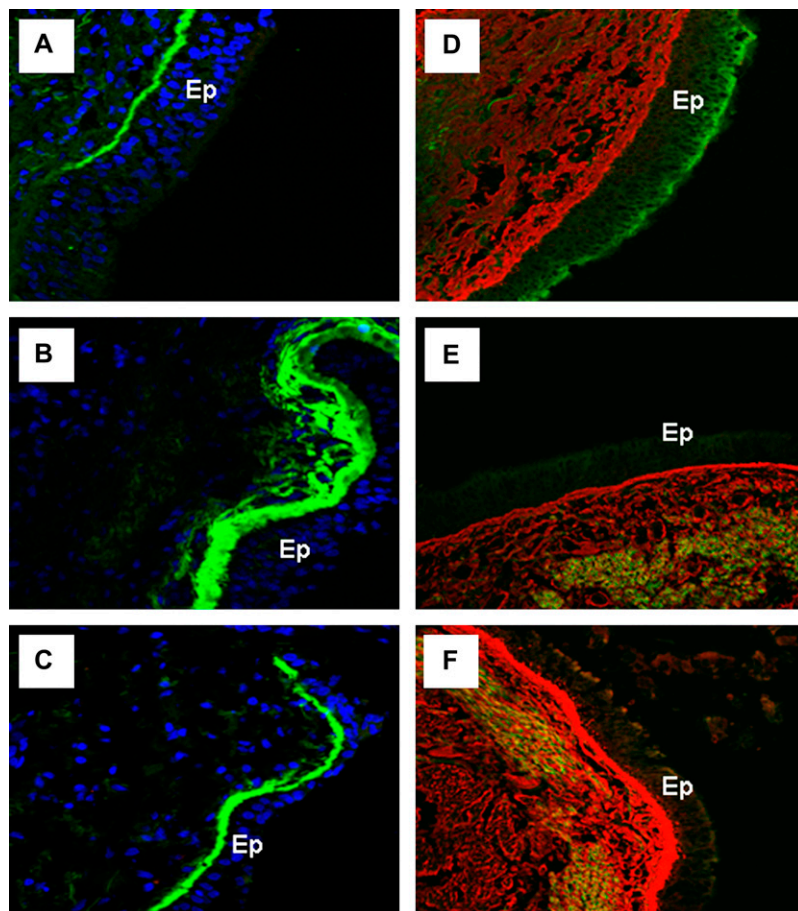


**Figure 4.** Representative photomicrographs of reticular basement membrane immunoreactivity for tenascin and procollagen III at baseline (A and D, respectively), 24 hours (B and E, respectively), and 7 days (C and F, respectively) after allergen challenge. Tenascin stained sections were counterstained with DAPI (4'-6'-diamidino-2-phenylindole) blue to provide orientation. The epithelium is marked Ep.

eosinophila (24). IL-13 blockade in an allergen-induced model of allergic asthma reversed established AHR despite the persistence of mucosal eosinophila (25).

ASM infiltration by mast cells may be an important determinant of AHR in asthma because it was a defining difference between asthma and eosinophilic bronchitis (21). In our study, no increase in mast cell numbers in either the total number or in the number associated with ASM was observed. Although these findings do not necessarily contradict a role for mast cells in baseline AHR, they do not support a role for this cell type in allergen-induced increases in AHR.

In an allergen-induced study by Gauvreau and associates on the cellular kinetics of cells in induced sputum (26), eosinophilia remained elevated 7 days after allergen inhalation challenge, albeit at levels considerably less than at 24 hours. This is in contrast to our findings of resolution of mucosal eosinophils to baseline levels. The reason for this difference between measures of airway luminal eosinophils and tissue cells is not clear but may reflect eosinophils that have been cleared from the submucosa but which can still be detected in the sputum at a time point when tissue infiltration has resolved.

Although we show that aspects of airway remodeling can be dissociated from airway inflammation, an important point from our study is that initial increases in cellular inflammation were associated with increased expression of remodeling markers and of activated (myo-) fibroblasts. Inflammation can be considered as a response to tissue injury. With acute injury, inflammation occurs with the purpose of carrying out restoration of the tissue to a normal state. Induction of airway tissue repair must occur rapidly and therefore will be expected to be in association with

inflammatory cell recruitment. Given that the induction of airway remodeling in response to allergen-induced injury is seen as early as 24 hours (8), it is possible that both eosinophil and macrophage infiltration seen in our model at 24 hours are responses related to airway repair (27).

Some aspects of remodeling, such as tenascin deposition, might be the consequence of inflammatory cell infiltration (13). As cellular inflammation resolved at 7 days, in parallel, RBM tenascin levels returned toward baseline values. Other aspects of remodeling may be initiated by inflammatory cell mediators acting directly on structural cells and leading to sustained activation and production of extracellular matrix (ECM) components, such as collagen. The significant correlation of HSP-47 with AHR at the 24-hour time point and the association of procollagen I expression at Day 7 are important as this suggests that individuals with increased AHR at the early time point display an enhanced capacity to generate collagen. Increases in AHR and collagen markers do not necessarily mean a cause-and-effect relationship, with other complex variables being operative. Nevertheless, the association of AHR with remodeling allows further hypotheses to be constructed and identifies remodeling markers that need further focus. The overall suggestion is that selected features of airway remodeling might be related to inflammatory cell sources of cytokine and growth factors, whereas other aspects may be initiated by inflammation but can later proceed independently of inflammation, possibly through structural cell activation.

The mechanisms by which remodeling may contribute to AHR remain an area of vigorous debate. Mathematical modeling predicts that any increase in airway wall thickness internal to the ASM layer will amplify the airway narrowing at the time of

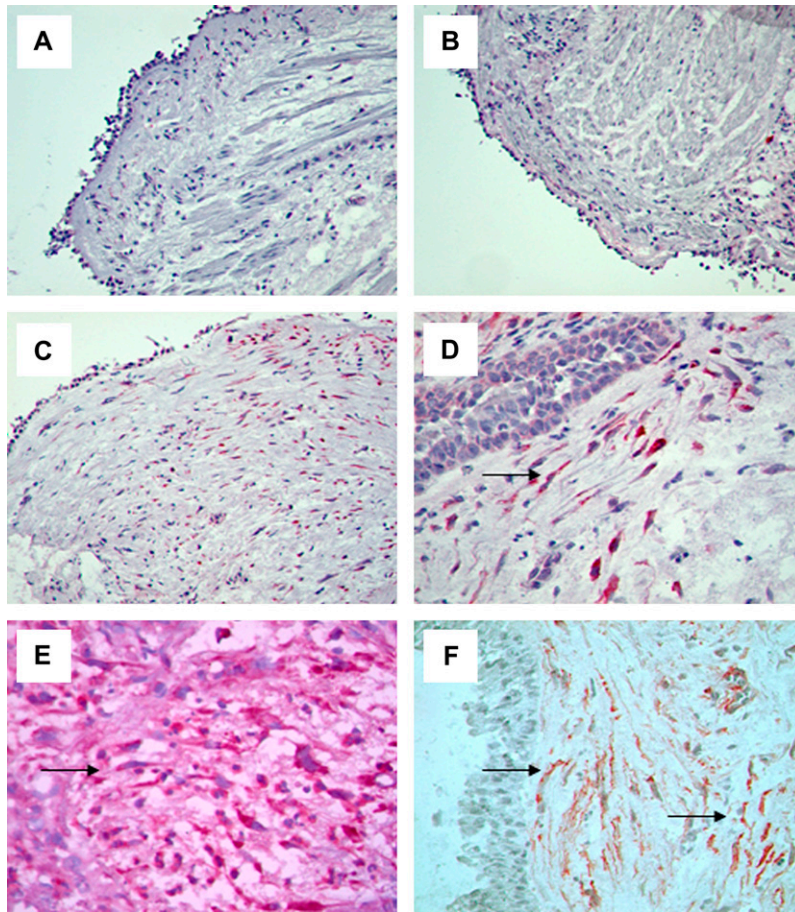


**Figure 5.** The expression of heat shock protein-47 (A) in fibroblast-like cells at baseline, 24 hours (B), and 7 days (C and D; original magnification  $\times 40$  and  $\times 100$ , respectively) after allergen challenge in a representative volunteer. Fibroblasts were identified morphologically as being fusiform in shape with elongated nuclei. Immunoreactive positive cells identified as leukocytes within this zone were not counted. Fibroblast-like cells staining positive for procollagen I (E) and  $\alpha$ -smooth muscle actin (F) are also presented (original magnification  $\times 100$  and  $\times 40$ , respectively).

ASM contraction; airways with increased ASM narrow to a much greater extent than airways with less smooth muscle volume for a given degree of circumferential smooth muscle shortening (28). Such predictions are important given that increased ASM mass is the only structural feature that distinguishes severe asthma from moderate disease (29). Greater ASM mass will not only lead to an excessive degree of muscle shortening (30) but also

to greater force generation, leading to a disproportionate reduction in airway patency for a given degree of ASM contraction (31). ASM has been found to encroach onto the RBM and epithelium in severe asthma (32), so that even minor contraction will affect airway narrowing. Such findings may explain the persistent AHR seen in asthma under basal conditions.

An obvious and important question is what component of the increased AHR is a result of inflammation and what aspect is the consequence of any early remodeling process. Inflammation may lead to priming of neurogenic mechanisms with consequent heightened neural reflexes on ASM, which must have a contributory factor, although at present there are few or no supporting data for such dysregulated neural mechanisms. Airway obstruction

**TABLE 3. SUMMARY OF INFLAMMATORY CELL NUMBERS IN BIOPSIES**

| Cell Type                                      | 0 Hours               | 24 Hours               | 7 Days              |
|------------------------------------------------|-----------------------|------------------------|---------------------|
| MBP <sup>+</sup> eosinophils                   | 4.7<br>(1.7–26.65)    | 36*<br>(23.25–58.5)    | 8<br>(4.5–22)       |
| CD68 <sup>+</sup> macrophages                  | 30.5<br>(2.65–42.7)   | 40.0*<br>(16.55–94.65) | 34.75<br>(21–57)    |
| CD3 <sup>+</sup> T cells                       | 27.30<br>(22.4–64)    | 86.60*<br>(55.7–107.3) | 43<br>(15.45–57.9)  |
| CD4 <sup>+</sup> T cells                       | 18.2<br>(2.85–31.30)  | 40.00<br>(21.5–60.45)  | 32.8<br>(3–39.70)   |
| CD8 <sup>+</sup> T cells                       | 2.4<br>(0.6–6.25)     | 14.20<br>(1.45–33.10)  | 4<br>(0–13.95)      |
| Elastase <sup>+</sup> neutrophils              | 22.0<br>(13.45–27.30) | 29.4*<br>(22–38)       | 21.15<br>(16.15–31) |
| Tryptase <sup>+</sup> mast cells               | 20.7<br>(11.6–34.85)  | 20.25<br>(4.75–35.65)  | 17.35<br>(9.3–25.9) |
| Tryptase <sup>+</sup> smooth muscle mast cells | 8<br>(2–15.65)        | 4<br>(1–14)            | 6<br>(0–20)         |

*Definition of abbreviation:* MBP = major basic protein.

The cell counts (cells/mm<sup>2</sup>) are expressed as the median (interquartile range).

\*  $p < 0.05$  compared with the cell count at baseline.

**TABLE 4. SUMMARY OF DATA ANALYSIS USING A MIXED MODELING APPROACH**

| Measure          | Change per Day | 95% CI        | p Value |
|------------------|----------------|---------------|---------|
| PC <sub>20</sub> | –0.15          | –0.26, –0.03  | 0.011   |
| FEV <sub>1</sub> | 0.37           | –0.62, 1.37   | 0.460   |
| MBP              | 1.18           | –4.15, 6.50   | 0.665   |
| CD68             | –0.48          | –5.56, 4.61   | 0.854   |
| CD3              | –2.64          | –6.88, 1.60   | 0.223   |
| Elastase         | 1.05           | –1.15, 3.24   | 0.351   |
| Tenascin         | 10.14          | –58.59, 78.86 | 0.772   |
| Procollagen III  | 140.20         | 63.78, 216.62 | < 0.001 |
| Procollagen I    | 19.16          | 7.09, 31.24   | 0.002   |
| HSP-47           | 28.71          | 14.19, 43.23  | < 0.001 |

*Definition of abbreviations:* CI = confidence interval; HSP = heat shock protein; MBP = major basic protein.

defines the late asthmatic response. Airway obstruction can be explained to a certain extent as a consequence of inflammatory cell infiltration with subsequent mediator and cytokine release. The late phase response in skin, in response to specific allergen injection into the dermis of atopic subjects, is seen as an edematous, red, and indurated area, which peaks at 6 to 9 hours and resolves within 24 to 48 hours. In the airway, similar edema and vascularity may occur during the LAR and may thus contribute to the fall in FEV<sub>1</sub> (33, 34). Although such a mechanism may exist, it is unlikely to be the major pathway leading to the transient increase in AHR as evidenced by an early study (35). Excessive mucus production will also contribute to the obstruction. Any airway narrowing will, at least in geometric terms, contribute to AHR, in that a narrowed airway will tend to narrow further to a lower dose of stimulus (a leftward shift in the curve for bronchoconstrictor dose-response). It is also possible that airway narrowing alone contributes to fibroblast activation because *in vitro* studies show induction of ECM production in fibroblasts by mechanical stretch (36, 37).

Our group and others have previously shown that patients with asthma at baseline express significantly more tenascin in the RBM of the airway and that tenascin is up-regulated further in response to allergen-induced airway injury, which is important (8, 13, 38). This is in contrast to the RBM in normal volunteers without asthma in whom there is minimal or no expression of tenascin. Although the up-regulation of tenascin after allergen may be related to the role of tenascin in inflammatory cell trafficking, it may be that, even at this stage, the increased deposition of ECM components, of which acute tenascin deposition is an example, may lead to increased airway narrowing and affect airway wall compliance, which contributes to AHR. It is probable that the expression of other ECM components may also be modulated, not only in the RBM but in the deeper layers of the submucosa (39).

Collagen III is the predominant collagen of the RBM (40). We have previously demonstrated that there is no significant increase in RBM expression of procollagen III at the 24-hour time point (8) and this again was the finding in the current study. However, at the 7-day time point, there was significantly increased procollagen III deposition in the RBM alongside the markedly increased HSP-47 and procollagen I synthesis in the submucosa. Again, it is tempting to speculate that such RBM increases may contribute to AHR via enhanced luminal narrowing and compliance-dependent effects.

Despite the association of RBM thickening with asthma and its correlation with AHR (41), the presence of RBM thickening in itself does not lead to AHR as evidenced by studies in eosinophilic bronchitis. One can speculate that the exact composition of ECM components in the lamina reticularis differs between asthma and eosinophilic bronchitis, or alternatively, that RBM thickness reflects modulation of ECM components in the deeper submucosa (39), which when in association with other remodeling changes in the submucosa, such as vascular changes and particularly ASM modulation (42) leading to excessive smooth muscle contractility, can influence AHR.

It is important to comment on the observations made in four single early responders (SERs). These individuals had no increased AHR or airway obstruction at either 24 hours or 7 days after allergen challenge, confirming previous observations (1, 2). The numbers of SERs in this study are too small for comparison with dual responders and therefore no firm assumptions can be made. However, the SERs did demonstrate nonsignificant increases in inflammatory cell recruitment at the 24-hour time point albeit to a markedly less degree than that found with the DAR group. This is consistent with previous studies (1, 26, 43). Three of the four volunteer SERs had no

increase in RBM tenascin or procollagen expression, whereas all four volunteers failed to demonstrate any increased expression of HSP-47 or procollagen in submucosal fibroblast-like cells.

Although increased collagen synthesis is initiated in response to inflammatory cell release of mediators such as transforming growth factor (TGF)- $\beta_1$  and IL-13, it appears that induction is further amplified and sustained in structural cells as evidenced by the predominant immunostaining of HSP-47 in fibroblast-like cells, at a time point when inflammation has returned to baseline values but AHR is sustained. It is possible that this aspect of airway remodeling contributes to sustained AHR at this time point.

Whether structural cell activation can occur in the absence of inflammation remains unanswered. Our previous data supported the hypothesis that the epithelial-mesenchymal trophic unit (EMTU), the embryologic unit driving airway development, which is suggested to be reactivated in airway remodeling (44), was rapidly activated in response to allergen with the TGF superfamily and IL-13 signaling (8). The activated epithelium is a significant source of TGF superfamily ligands and it is possible that remodeling events may proceed in the absence of inflammation through EMTU activation alone.

A therapeutic implication from this study is that measuring the degree of inflammation does not allow insight into disease severity in terms of AHR. This may explain why several studies with antiinflammatory inhaled steroid therapy have failed to abolish AHR despite reductions in cellular inflammation (45–48). Our observations may also help to explain several clinical observations relating to therapeutic responsiveness. For example, in the CAMP (Childhood Asthma Management Program) research group study (49), the benefits of inhaled corticosteroid therapy on post-bronchodilator improvements were only observed for the first 3 years. A similar lack of effectiveness of inhaled steroids on the natural history of asthma, measured in terms of post-bronchodilator FEV<sub>1</sub>, despite initial improvements in asthma outcome was observed in the START (Steroid Treatment As a Regular Therapy in early asthma) study (50). These findings may be explained by the efficacy of steroids in decreasing inflammation-related remodeling that leads to initial improvement in FEV<sub>1</sub>. The observation that this improvement is lost after 3 years could be explained by epithelial-mesenchymal-driven remodeling events that are considered refractory to corticosteroids (51, 52). The persistence of troublesome AHR despite high-dose inhaled corticosteroid in patients with moderate-severe asthma is an important clinical problem and is currently being addressed by the addition of long-acting  $\beta_2$ -agonists, which effectively target airway smooth muscle, a key aspect of airway remodeling.

The difficulty in performing mechanistic studies of airway remodeling in humans has led to insight from several animal models of disease (53). Animal studies have suggested that structural changes can be associated with AHR in the absence of cellular inflammation. Leigh and colleagues (54) were able to show in a chronic allergen setting that, despite the resolution of inflammation early on after the cessation of allergen exposure, changes in AHR and remodeling remain associated for at least 8 weeks after the final allergen exposure. Interestingly, although acute allergen exposure-induced increase in AHR was associated with increased IL-13 levels, AHR that persisted beyond the inflammatory stages was not associated with increased IL-13. This led the authors to conclude that, although early cellular inflammatory events with associated Th2 cytokines contribute to the initiation of remodeling events, sustained AHR is a consequence of increased airway contractile tissue.

In summary, persistent inflammatory cell infiltration of the bronchial mucosa does not appear to be essential to sustain

allergen-provoked increases in AHR, which are, however, associated with persistent activation of airway remodeling. The sustained AHR, however, could reflect persistence of end effects of inflammation on mucosal remodeling processes, which, in turn, have downstream effects contributing to AHR. It is also possible that there are mechanisms for AHR in response to allergen challenge that are not due to airway inflammation and may involve direct remodeling events that are not inflammatory cell dependent. This might explain why inhaled corticosteroid therapy in asthma reduces but does not abolish AHR despite a dramatic reduction in inflammation. The development of further longitudinal studies in humans with asthma will be important to provide further insight into such questions.

**Conflict of Interest Statement:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

**Acknowledgment:** The authors thank the staff of the Bronchoscopy Unit at the Royal Brompton Hospital for their assistance with the study. They also thank Michael Roughton, NHLI Division, Imperial College London, for statistical advice.

## References

- Dorman SC, Sehmi R, Gauvreau GM, Watson RM, Foley R, Jones GL, Denburg JA, Inman MD, O'Byrne PM. Kinetics of bone marrow eosinophilopoiesis and associated cytokines after allergen inhalation. *Am J Respir Crit Care Med* 2004;169:565–572.
- Cockcroft DW, Ruffin RE, Dolovich J, Hargreave FE. Allergen-induced increase in non-allergic bronchial reactivity. *Clin Allergy* 1977;7:503–513.
- Robinson D, Hamid Q, Bentley A, Ying S, Kay AB, Durham SR. Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol* 1993;92:313–324.
- Metzger WJ, Richerson HB, Worden K, Monick M, Hunninghake GW. Bronchoalveolar lavage of allergic asthmatic patients following allergen bronchoprovocation. *Chest* 1986;89:477–483.
- Beasley R, Roche WR, Roberts JA, Holgate ST. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 1989;139:806–817.
- Dorman SC, Efthimiadis A, Babirad I, Watson RM, Denburg JA, Hargreave FE, O'Byrne PM, Sehmi R. Sputum CD34+IL-5Rα+ cells increase after allergen: evidence for *in situ* eosinophilopoiesis. *Am J Respir Crit Care Med* 2004;169:573–577.
- Diaz P, Gonzalez MC, Galleguillos FR, Ancic P, Cromwell O, Shepherd D, Durham SR, Gleich GJ, Kay AB. Leukocytes and mediators in bronchoalveolar lavage during allergen-induced late-phase asthmatic reactions. *Am Rev Respir Dis* 1989;139:1383–1389.
- Phipps S, Benyahia F, Ou TT, Barkans J, Robinson DS, Kay AB. Acute allergen-induced airway remodeling in atopic asthma. *Am J Respir Cell Mol Biol* 2004;31:626–632.
- Kariyawasam HH, Aizen M, Barkans J, Robinson DS, Kay AB. Persistence of airway hyper-responsiveness (AHR) after resolution of bronchial mucosal inflammation following allergen inhalation challenge in mild asthmatics [abstract 1086]. *J Allergy Clin Immunol* 2006;117:S158.
- Crapo RO, Casaburi R, Coates AL, Enright PL, Hankinson JL, Irvin CG, MacIntyre NR, McKay RT, Wanger JS, Anderson SD, *et al.* Guidelines for methacholine and exercise challenge testing—1999. *Am J Respir Crit Care Med* 2000;161:309–329.
- Taylor DA, Harris JG, O'Connor BJ. Comparison of incremental and bolus dose inhaled allergen challenge in asthmatic patients. *Clin Exp Allergy* 2000;30:56–63.
- Hamid Q, Azzawi M, Ying S, Moqbel R, Wardlaw AJ, Corrigan CJ, Bradley B, Durham SR, Collins JV, Jeffery PK. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J Clin Invest* 1991;87:1541–1546.
- Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, Barnes N, Robinson D, Kay AB. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 2003;112:1029–1036.
- Brusasco V, Crimi E, Gianiorio P, Lantero S, Rossi GA. Allergen-induced increase in airway responsiveness and inflammation in mild asthma. *J Appl Physiol* 1990;69:2209–2214.
- Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998;157:4–9.
- Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, Holgate ST. Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Respir Dis* 1990;142:863–871.
- Ollerenshaw SL, Woolcock AJ. Characteristics of the inflammation in biopsies from large airways of subjects with asthma and subjects with chronic airflow limitation. *Am Rev Respir Dis* 1992;145:922–927.
- Iredale MJ, Wanklyn SA, Phillips IP, Krausz T, Ind PW. Non-invasive assessment of bronchial inflammation in asthma: no correlation between eosinophilia of induced sputum and bronchial responsiveness to inhaled hypertonic saline. *Clin Exp Allergy* 1994;24:940–945.
- Brusasco V, Crimi E, Pellegrino R. Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax* 1998;53:992–998.
- Brightling CE, Symon FA, Birring SS, Bradding P, Wardlaw AJ, Pavord ID. Comparison of airway immunopathology of eosinophilic bronchitis and asthma. *Thorax* 2003;58:528–532.
- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;346:1699–1705.
- McMillan SJ, Lloyd CM. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin Exp Allergy* 2004;34:497–507.
- Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, *et al.* A critical role for eosinophils in allergic airways remodeling. *Science* 2004;305:1776–1779.
- Siegle JS, Hansbro N, Herbert C, Yang M, Foster PS, Kumar RK. Airway hyperreactivity in exacerbation of chronic asthma is independent of eosinophilic inflammation. *Am J Respir Cell Mol Biol* 2006;35:565–570.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258–2261.
- Gauvreau GM, Watson RM, O'Byrne PM. Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. *Am J Respir Crit Care Med* 1999;160:640–647.
- Kay AB, Phipps S, Robinson DS. A role for eosinophils in airway remodelling in asthma. *Trends Immunol* 2004;25:477–482.
- Moreno RH, Hogg JC, Pare PD. Mechanics of airway narrowing. *Am Rev Respir Dis* 1986;133:1171–1180.
- Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;167:1360–1368.
- Wiggs BR, Moreno R, Hogg JC, Hilliam C, Pare PD. A model of the mechanics of airway narrowing. *J Appl Physiol* 1990;69:849–860.
- Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol* 1993;74:2771–2781.
- Madison JM. Migration of airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2003;29:8–11.
- Hogg JC. Bronchial mucosal permeability and its relationship to airways hyperactivity. *J Allergy Clin Immunol* 1981;67:421–425.
- Boucher RC, Pare PD, Hogg JC. Relationship between airway hyperactivity and hyperpermeability in *Ascaris*-sensitive monkeys. *J Allergy Clin Immunol* 1979;64:197–201.
- O'Byrne PM, Dolovich M, Dirks R, Roberts RS, Newhouse MT. Lung epithelial permeability: relation to nonspecific airway responsiveness. *J Appl Physiol* 1984;57:77–84.
- Chiquet M, Renedo AS, Huber F, Fluck M. How do fibroblasts translate mechanical signals into changes in extracellular matrix production? *Matrix Biol* 2003;22:73–80.
- Ludwig MS, Ftouhi-Paquin N, Huang W, Page N, Chakir J, Hamid Q. Mechanical strain enhances proteoglycan message in fibroblasts from asthmatic subjects. *Clin Exp Allergy* 2004;34:926–930.
- Laitinen A, Altraja A, Kampe M, Linden M, Virtanen I, Laitinen LA. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 1997;156:951–958.
- Kasahara K, Shiba K, Ozawa T, Okuda K, Adachi M. Correlation between the bronchial subepithelial layer and whole airway wall thickness in patients with asthma. *Thorax* 2002;57:242–246.
- Wilson JW, Li X. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clin Exp Allergy* 1997;27:363–371.

41. Boulet LP, Laviolette M, Turcotte H, Cartier A, Dugas M, Malo JL, Boutet M. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. *Chest* 1997;112:45-52.
42. Black JL, Roth M, Lee J, Carlin S, Johnson PR. Mechanisms of airway remodeling: airway smooth muscle. *Am J Respir Crit Care Med* 2001;164:S63-S66.
43. Wood LJ, Inman MD, Watson RM, Foley R, Denburg JA, O'Byrne PM. Changes in bone marrow inflammatory cell progenitors after inhaled allergen in asthmatic subjects. *Am J Respir Crit Care Med* 1998;157:99-105.
44. Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol* 2000;105:193-204.
45. Juniper EF, Kline PA, Vanzieleghe MA, Ramsdale EH, O'Byrne PM, Hargreave FE. Effect of long-term treatment with an inhaled corticosteroid (budesonide) on airway hyperresponsiveness and clinical asthma in nonsteroid-dependent asthmatics. *Am Rev Respir Dis* 1990;142:832-836.
46. Lundgren R, Soderberg M, Horstedt P, Stenling R. Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. *Eur Respir J* 1988;1:883-889.
47. Duddridge M, Ward C, Hendrick DJ, Walters EH. Changes in bronchoalveolar lavage inflammatory cells in asthmatic patients treated with high dose inhaled beclomethasone dipropionate. *Eur Respir J* 1993;6:489-497.
48. Adelroth E, Rosenhall L, Johansson SA, Linden M, Venge P. Inflammatory cells and eosinophilic activity in asthmatics investigated by bronchoalveolar lavage: the effects of antiasthmatic treatment with budesonide or terbutaline. *Am Rev Respir Dis* 1990;142:91-99.
49. The Childhood Asthma Management Program Research Group. Long-term effects of budesonide or nedocromil in children with asthma. *N Engl J Med* 2000;343:1054-1063.
50. Pauwels RA, Pedersen S, Busse WW, Tan WC, Chen YZ, Ohlsson SV, Ullman A, Lamm CJ, O'Byrne PM. Early intervention with budesonide in mild persistent asthma: a randomised, double-blind trial. *Lancet* 2003;361:1071-1076.
51. Ward C, Walters H. Airway wall remodelling: the influence of corticosteroids. *Curr Opin Allergy Clin Immunol* 2005;5:43-48.
52. Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, Boulet LP, Hamid Q. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003;111:1293-1298.
53. Kariyawasam HH, Robinson DS. Airway remodelling in asthma: models and supermodels? *Clin Exp Allergy* 2005;35:117-121.
54. Leigh R, Ellis R, Wattie J, Southam DS, De Hoogh M, Gauldie J, O'Byrne PM, Inman MD. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 2002;27:526-535.